University of Alberta

Extraction of Carbohydrates and Phenolics from Barley Hull using Pressurized/Subcritical Fluids

by

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A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfillment of the requirements for the degree of

Master of Science

in

Bioresource and Food Engineering

Department of Agriculture, Food and Nutritional Science

Spring 2013

Edmonton, Alberta

I lovingly dedicate this thesis to my father, who always stood behind me and believed that I would succeed. Gone now but never forgotten. I will miss you always and love you forever.

It is also dedicated to my mother, who was constantly supporting me through this difficult time.

ABSTRACT

Canada is one of the largest barley producers, generating barley hull which accounts for 10-13% wt of the grain. Barley hull has valuable components, such as carbohydrates and phenolics. This thesis studied the extraction of total carbohydrates and phenolics from the hull of a new barley variety (*BT 584)* using solid-liquid batch (SLB), subcritical fluid (sCF) and supercritical CO_2 (SC-CO_2) extraction methods. The optimization of the variables was performed using response surface methodology (RSM) with a central composite design. The sCF extraction was the most effective method, yielding 122.4 mg of total phenolics/g of barley hull and 589.4 mg of total carbohydrates/g of barley hull at 240°C, 15MPa, with 12% ethanol concentration, flowing at 5 mL/min. A kinetic model was used to fit the curves of the sCF extraction with mean square error lower than 0.06.

Keywords*: Total phenolics, total carbohydrates, barley hull, extraction, supercritical CO_2, pressurized fluids.*

ACKNOWLEDGEMENTS

It is an immense pleasure to thank many people who made this thesis possible. It has been a great privilege to spend a couple of years in the Department of Agriculture, Food and Nutritional Science at University of Alberta, and this experience will be fruitful to me.

Foremost, my debt of gratitude must go to my supervisor, Dr. Marleny D. A. Saldaña. She was always patient in providing me the vision, encouragement, and guidance throughout the research and helped me to complete this dissertation effectively. She has been a very strong and supportive advisor to me throughout my graduate studies, but she has also provided me freedom to pursue independent work.

Besides my advisor, I would like to extend my special thanks to my committee member, Dr. Thava Vasanthan for his constant support, and helpful suggestions. His guidance has served me well and I owe my heartfelt appreciation for him.

I am indebted to my lab colleagues for providing a stimulating and fun environment for learning and growing. I am especially grateful to Dr. Victor Alvarez and Jessica Cahyadi for their constant help with my experiments and analysis. I take the opportunity to thank Ivor, Metzeri, Sergio, Anupam, Ehsan, Glaucia, Deniz, Ozan, and Sunny for their constant motivation and suggestions. I also want to extend my special gratitude to Dr. Feral Temelli for her time to time encouragement.

A special thank is extended to my best friends Bhuvanvir Maingi, Anjali Gupta, Ritesh Kaur Saini, Neha Khanna and Santu Mandal for helping

me through the difficult times, and all the emotional support, camaraderie, entertainment and caring they provided. I also want to thank my extended family and friends for providing a supportive environment for me. My mother, brother and sister-in-law particularly always loved, cared and supported me.

Lastly, and most importantly, I would like to thank Alberta Agriculture (Lacombe, AB, Canada) for providing the barley sample and to Natural Sciences and Engineering Research Council of Canada (Dr. Saldaña's NSERC) for providing the funds for carrying out this research.

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NOMENCLATURE

Symbols	**Name**
T	Temperature
P	Pressure
RI	Refractive index
κ	Conductivity
L	Hunter color index *L*
a	Hunter color index *a*
b	Hunter color index *b*

ABBREVATIONS

SD	Standard deviation
sCF	Subcritical fluid extraction
SC-CO_2	Supercritical carbon dioxide
DPPH	2,2-diphenyl-1-picrylhydrazyl method
FRAP	Ferric reducing/Antioxidant power method
RSM	Response Surface Methodology
IL	Ionic liquid
SCW	Subcritical water
CCD	Central composite design
ANOVA	Analysis of variance
CHO	Total carbohydrates
Phe	Total phenolics

Chapter 1: Introduction

1.1. Rationale

Barley (*Hordeum vulgare*) is an ancient and important cereal grain, which has been domesticated primarily as a feed and malting grain (Baik and Ullrich, 2008). Canada is the fifth largest producer of barley in the world, predominantly being grown in the Prairie Provinces of Alberta, Manitoba, and Saskatchewan. In 2010-2011, the annual production of barley in Canada was 7.7 M mt (Statistics Canada, 2010), out of which over half of the total amount of barley was grown in Alberta (Alberta Barley Commission, 2010).

The extraction of phenolic compounds from plant biomass is the first step in the utilization of phytochemicals in the preparation of nutraceuticals, food ingredients, and cosmetic products. Barley grain contains a wide range of phenolic compounds, including benzoic and cinnamic acid derivatives, anthocyanins, proanthocyanidins, lignans, etc (Jende-strid, 1993; Goupy et al., 1999; Quinde-Axtell and Baik, 2006). Ferulic acid is the major free phenolic acid in barley grain and bran while the predominant bound phenolic acids detected in barley grain after acidic and enzymatic treatments are *p*-hydroxybenzoic acid (Naczk and Shahidi, 2006).

Most of the barley-based bread and soup products in the market are prepared from the dehulled barley grain as the hull is considered indigestible by humans (Cruz et al., 2007; Garrote et al., 2008). However, some studies have reported that barley hull, which constitutes about 10-13% of the whole grain is a great source of phenolics as well as carbohydrates (Nordkvist et al.,

1984; Hernanz et al., 2001; Höije et al., 2005). Höije et al. (2005) reported a total carbohydrate content of 87% in the hull of Cindy or Waxy variety of barley grain. Barley hull can thus be utilized as a biomass as it has a high content of lignocellulosic (LC) material, approximately 31-34% cellulose, 24-29% hemicelluloses and 14-15% lignin content. Barley hull can thus be further utilized as a source for fermentable sugars for the production of biofuel (e.g. ethanol production). Moreover, phenolic acids are also mainly concentrated in the hull portion of the barley grain (Nordkvist et al., 1984; Hernanz et al., 2001). Hao and Beta (2012) showed that 1kg of barley hull contains 10.71g total phenolics as measured according to the ferulic acid equivalent. In addition, Waldron et al. (1996), and Bunzel et al. (2004) reported that ferulic acid and *p*-coumaric acid are associated with the cell wall constituents as they are ester-linked to them, especially with the arabinoxylans and lignin.

Phenolics have proved to contribute to the antioxidant activity of foods (Parr and Bolwell, 2000). Further research on phenolics showed their additional benefits because of their anti-allergic, anti-inflammatory, anti-microbial, cardioprotective and anti-thrombotic effects (Benavente-Garcıa et al., 1997; Middleton et al., 2000; Manach et al., 2005). Due to these benefits, aqueous solutions of methanol, ethanol and acetone separately or in combination have been used to maximize the extraction of phenolics from barley flour (Sosulski et al., 1982; Velioglu et al., 1998; Miller et al., 2000; Zieliński and Kozłowska, 2000). Bonoli et al. (2004) reported the use of 4:1 ratio of aqueous ethanol, aqueous methanol, and aqueous acetone for the extraction of free phenolics from barley flour. It was estimated that the use of 4:1 ratio of aqueous acetone was able to extract the highest amount of total

phenolics content of barley flour (0.68 mg/g) after 40 min of extraction. However, these methods involve the use of toxic petrochemical solvents and/or long extraction times ranging from 1to 24 h (Thondre et al., 2010; Sharma and Gujral, 2010; Madhujith and Shahidi, 2009; Hernanz et al., 2001 etc.) and temperature (from room temperature to 100°C) that might accelerate the oxidation of total phenolics (Robards, 2003) unless a reducing agent such as cysteine is added to the solvent (Khanna et al., 1968; Richard et al., 1991). Therefore, there is a need to use a green process which could provide a high yield in short time, minimizing degradation.

Supercritical CO_2 (SC-CO_2) is a green solvent which is commonly used in food and pharmaceutical applications. Colombo et al. (1998) reported that SC-CO_2 at 40°C and 23.7 MPa extracted 30-50% higher amounts of tocopherols and tocotrienols from barley grain than with the *n*-hexane Soxhlet extraction. Other cereal crops, such as triticale, rye, wheat, rice, etc. have been treated with SC-CO_2 to extract alkylresorcinols (Landberg et al., 2007; Dey and Mikhailopulo, 2009; Athukorala et al., 2010), tocochromanols (Fratianni et al., 2002). Apart from the phenolic compounds extraction from different cereal crops, the SC-CO_2 has also shown to be an effective drying method to prepare aerogels from barley ß-glucan (Comin et al., 2012). During this process, barley ß-glucan aerogels were prepared using supercritical CO_2 at 15MPa, 40°C and the CO_2 flowed at 1mL/min for approximately 4h to achieve the desired goal.

Another green technology recently explored is subcritical water extraction that is carried out using hot water at temperatures between 100 and 374°C under high enough pressure to maintain the water in its liquid state and

therefore they are also known as pressurized fluids. Thus, a subcritical fluid (sCF) relies on the decrease of fluid polarity and the dielectric constant and an increase on the ion product with an increase in temperature.

Some common examples of sCF are water and aqueous ethanol that have already been used for the extraction of phenolics from food by-products of plant origin, such as cinnamon bark (Khuwijitjaru et al., 2012), apple pomace (Wijngaard and Brunton, 2009), pomegranate seed residues (He et al., 2012), defatted rice bran (Chiou et al., 2012), potato peel (Singh and Saldaña, 2011), and others. Most of the studies dealing with sCF extraction of phenolic acids from different matrices have used a temperature range of 100-220°C at 6-10MPa with 2-3mL/min flow rate of the solvent (Khuwijitjaru et al., 2012; He et al., 2012; Chiou et al., 2012; Singh and Saldaña, 2011; Fabian et al., 2010; Srinivas et al., 2010; Hassasroudsari et al., 2009). Hassasroudsari et al. (2009) obtained a high amount of total phenolics (17.7 mg of sinnapic acid equivalent/g of canola meal) using 95% ethanol at subcritical conditions. The extraction was carried out at a pressure of 6.9 MPa with 1 mL/min of solvent flowing for 30 min achieving the maximum antioxidant activity of 3.6-4.4 μmol Trolox equivalent/g of extract) as measured using Trolox equivalent antioxidant capacity (TEAC) assay and 92-95% using ß-carotene-linolenic acid assay, respectively. Hydrolysis reactions of rice bran and soya bean meal using subcritical water at 200-220°C and 4MPa have produced value-added soluble products, such as proteins, amino acids and reducing sugars (Watchararuji et al., 2008).

Even though the use of subcritical fluids had an advantage of short time for the extraction, some Maillard reactions, such as non-enzymatic links

between the carbonyl group of reducing carbohydrates and the amino group of free amino acids as well as lysyl residues in proteins cannot be avoided. This reaction contributes to the non-enzymatic browning in food that may have either beneficial or detrimental effects (Friedman, 1996). Resnik and Chirife (1979) reported that the measurements of the extract solutions at absorbances of 294 and 420 nm can be related to the formation of aldehydes and browning products, respectively. These measurements also provide a better indication of the browning quality of the samples.

Although recent studies have demonstrated the potential of sCFs, only one study attempted the extraction of barley polysaccharides (β-glucan) and antioxidant compounds from barley grain to produce a functional drink known as barley tea (Kulkarni et al., 2008). Therefore, this study was conducted to evaluate the ability of the solid-liquid batch (aqueous ethanol), SC-CO_2 +aqueous ethanol as a co-solvent, and sCF (pressurized aqueous ethanol or aqueous ionic liquid) extraction methods to obtain total phenolics and total carbohydrates from *BT 584* barley hull. The extraction yields for each method were evaluated using spectrophotometric measurements. In addition, the antioxidant activity of the extract solutions was quantified by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and Ferric Reducing/Antioxidant Power (FRAP) analysis. Furthermore, some of the Maillard reaction products of the extracts were quantitatively measured after the extraction experiments.

In addition to the studies of the sCF using aqueous ethanol as a solvent for the extraction, a new solvent of aqueous N-methyl-2-hydroxyethylammonium acetate under subcritical conditions for the extraction of total carbohydrates and total phenolics were also studied.

1.2. Hypothesis

- Subcritical fluid extraction can increase the removal of total phenolics and total carbohydrates from barley hull.
- If Maillard reactions occur during subcritical fluid extraction then the dark color of the extracts can be related to their antioxidant activity of the samples.

1.3. Thesis objectives

The main objective of this thesis was to extract maximum amounts of total carbohydrates and total phenolics from barley hull using pressurized/subcritical fluid extraction. To achieve this main objective, some specific objectives were:

a) Identify and optimize process parameters for solid-liquid extraction

b) Identify and optimize process parameters for the SCW extraction and pressurized fluids extraction.

c) Compare the amounts of total carbohydrates and total phenolics obtained using solid-liquid batch aqueous ethanol extraction, pressurized fluids extraction and supercritical CO_2 + ethanol extraction.

Chapter 2: Literature Review

2.1. Major crops

Cereal and oilseeds are the most important agricultural crops grown in Canada with a total production of 66 million tonnes (Mt) during the year 2011 (Statistics Canada, 2011). In 2011, the highest total production of crops in Canada was due to the increase in the production of spring and durum wheat, barley, oat, canola and dry pea. Canada produced approximately 22.2 Mt of wheat which was the major crop grown. Among the other cereal crops, barley and oats were harvested in Canada to a greater extent in the range of 7.8 Mt and 3Mt, respectively. In addition, Canola oilseed production was 14.2 Mt. and about 2 Mt of dry pea was produced. (Statistics Canada, 2011).

2.2. Barley

2.2.1. Production

Barley (*Hordeum vulgare L.*), from the *Triticeae* tribe of the grass family Poaceae (Graminae), is one of the most harvested cereal crops in Canada. Canada is the fifth largest producer of barley, approximately 7.8 Mt was produced in 2011 (Statistics Canada, 2011). Over 90% of the barley grown in Canada is harvested in the western prairies, such as Alberta, Saskatchewan and Manitoba (USDA Foreign Agriculture Services). According to Statistics Canada (2011), barley production in the prairies has already averaged near 11.1Mt in recent years. Out of all the barley produced in Canada, about 2 Mt is selected for the malt industry while almost 9 Mt is being utilized as animal feed. The food industry uses only 13.5 thousand

tonnes of barley for the production of bread, soups, pasta, etc (Statistics Canada, 2011).

2.2.2. Structure and classification

A longitudinal structure of barley grain is presented in Fig. 2.1. The structure of barley grain is very similar to other cereal crops (wheat or oat). The barley grain is one-seeded which is called the caryopsis. Hull of the grain, which is composed of epidermis, fiber and parenchyma, constitutes about 10-13% of the barley weight. The hull covers the caryopsis, which includes the different layers of the grain known as bran, endosperm, and germ. These layers are made up of sub-layers, which are highlighted in Fig. 2.1. The total weight of the barley grain kernel ranges from 32-40mg. The endosperm is the main part of the grain and is mainly composed of granular starch embedded in protein matrix. Barley is classified into different categories: spring or winter type, two or six-row, and hulled or hulless depending on the presence or absence of hull attached to the grain, and also depending on the basis of their end-use for malting or for animal feed. Further classification of barley hull depends on its grain composition, being classified into normal, waxy or high amylose starch type, high β-glucan, high lysine and proanthocyanidin-free (Baik and Ullrich, 2008).

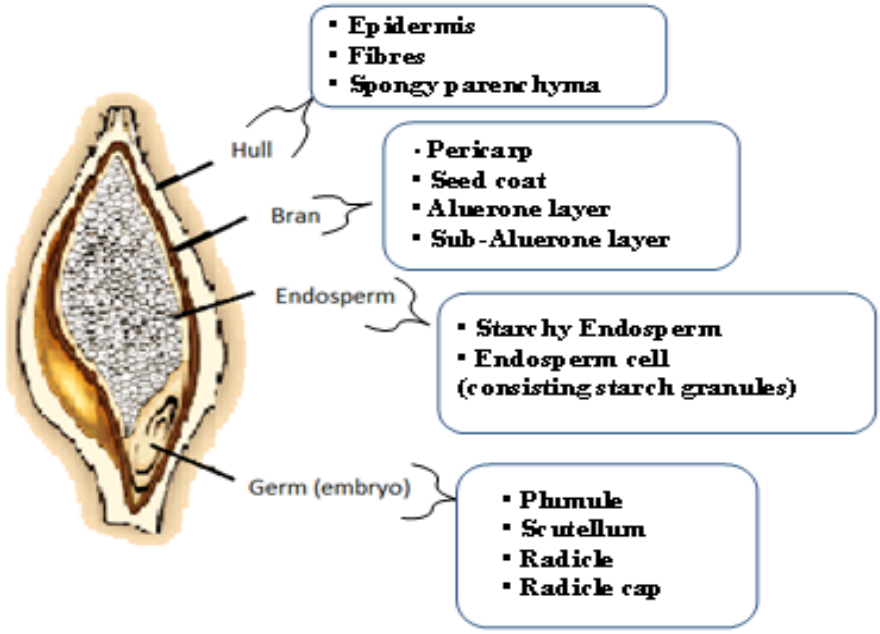


Figure 2.1 Longitudinal structure of barley grain with sub-layers (Adapted from Hoover and Vasanthan, 2009).

2.2.3. Uses of barley

The main uses of barley are in the malting and brewing industry for the production of beer and whiskey, animal feed and human food consumption. In Canada, about 83% barley produced barley is used as animal feed and only 17% is for the food (bread, soup, flour etc.) and industrial use (malting and brewing industry) (Statistics Canada, 2000-2008). barley demand for food and malting has increaesd in the last five to ten years as a result of increased health awareness and favorable market prices (Baik and Ullrich, 2008). The use of barley in the nutraceutical industry has emerged lately due to its high β-glucan content (Delaney et al., 2003). Vasanthan and Temelli (2008) reported that Polycell Technologies (USA), Cargill (USA) and GraceLinc Ltd. (New Zealand) are the companies which are currently producing barley β-glucan concentrates. Also, industrial applications of high

amylose barley starches in malting and brewing are studied for the diversification of barley utilization (Ganeshan et al., 2008).

2.2.3.1 Malting and brewing industry

Approximately 12% of the barley is used as malt for brewing beer. For malting purposes, the cultivars include hulled and hulless varieties but the hulled barley is usually preferred for its contribution to flavor and aids filtering during the brewing process (Gunkel et al., 2002). The physical, chemical and biochemical properties of barley grain influenced the malting process and the quality of the beer. The physical characteristics, such as germination percentage, germ growth, maturity of the kernel, and size are some of the factors that affect the malting process. The chemical composition of the grain such as starch, protein, β-glucan and their interactions affects the grain hardness, which decreases the malt extract yield (Psota et al., 2007). For malting, usually the soft variety of barley (Gupta et al., 2010) with 10.5% - 13.0% protein content for the six-row and two-row varieties is preferred (Dusabenyagasani et al., 2003). On the other hand, barley variety with high protein concentration of more than 15% is not suitable for the malting purpose as it requires a longer steeping time, has unreliable germination and produces low malt extract (Swanston et al., 2001). The discolored barley grain is also unsuitable for malting due to undesirable flavors produced during beer production due to the breakdown of phenolics during the mashing process (Mussatto et al., 2006). Thus, a successful malting barley export market demands the proper selection of cultivars with appropriate malting characteristics.

2.2.3.2 Human health and nutrition

Approximately 2% of the global barley production is used as food (Baik and Ullrich, 2008). The preferred barley used as food is the one which is thin-hulled, bright yellow-white, medium-hard and uniform in size. Barley is nutritionally rich due to its high carbohydrate content, high dietary fiber content, moderate protein content and a good source of phosphorous, selenium, manganese and copper (Ames et al., 2006). The dehulled, polished, and milled barley is often used to make porridge and soups, and as a substitute for rice in some of the Asian countries (Baik and Ullrich, 2008). In India and other surrounding countries, a considerable amount of barley is used for the production of baked foods, such as bread, noodles, and pilaf. It has been reported that barley foods have several positive effects on the human digestive system as their consumption reduces the transit time of the fecal matter, which is associated with a lower frequency of colon cancer and hemorrhoids (Tsai et al., 2004). A healthy colon is maintained by the fermentation of barley's soluble dietary fiber into short-chain fatty acids, such as butyric acid that could be absorbed and helps in inhibiting the hepatic cholesterol synthesis (Behall et al., 2004). Moreover, the production of other fermentation products such as propionic and acetic acids also provides fuel for liver and muscle cells (Liu, 2004). One of the most important dietary fibers produced of barley is the soluble glucan polymer β-glucan. The presence of β-glucan in diets increases the viscosity of foods during digestion, which leads to low amount of glucose absorption and reduction of blood glucose level, also known as glycemic index (Jenkins et al., 2007). These foods with low glycemic index are preferred to decrease the risk of diabetes in humans. Barley fibre is also a good source of

niacin, which is a B-vitamin that is known to reduce the blood clots and lowers the levels of total cholesterol. Thus, niacin has shown to be effective against cardiovascular diseases (Jood and Kalra, 2001). Due to the various health claims associated with barley grain utilization, nowadays barley based food products are aimed at the control of blood sugar levels in diabetic patients, and also to reduce cholesterol as well as heart diseases. Due to its low glycemic index, barley is also used by athletes who require a slow release of glucose in the blood.

2.2.3.3 Animal feed

Barley is often used as animal feed as its nutritive value is lower than that of corn or wheat. The two different varieties of barley, namely two-row and six-row hulless barley, which are produced in Canada for feed have relatively high protein content (14-15%). But, due to the high carbohydrate content, the two-row grain is preferred (Fregeau-Reid et al., 1992). Although, barley is a popular animal feed, grain with a high starch concentration is not appropriate for ruminants. This is due to the pH drop which is caused by rapid starch fermentation, which in turn reduces fiber digestion and causes digestive disorders. In addition, when high starch barley is used as feed for lactating cows, the milk fat content decreased (Larsen et al., 2009). It is therefore important to consider the grain composition when selecting the barley grain for animal feed.

2.2.4. Composition of barley kernel

Barley kernel is composed of hull (10-13% of kernel weight), bran (~14% of kernel weight), endosperm (~83% of kernel weight) and germ

(~2.5% of kernel weight) as shown in Fig 2.1. The endosperm is the most important component of barley kernel, consisting of 70-77% carbohydrates, 12-16% protein, along with less than 2% of vitamins (niacin, thiamine, etc.) and minerals (selenium, iron, magnesium, zinc, phosphorous and copper). In addition, the germ, which includes the embryo contains high quantity of protein (12-20%), followed by an appreciable amount of lipids (1.5-5%), B-complex vitamins and trace minerals (<2.5%). The bran is mainly composed of approximately 3% of protein, with 3-5% trace minerals (iron, zinc, manganese, etc.), B-vitamins (3-6%) and about 4.5-15% non-starch carbohydrates, which provides the dietary fiber (USDA Nutrient Database). Barley bran is composed of hull, pericarp, testa, and the aleurone layer (Chakraverty et al., 2003).

2.3. Barley Hull

2.3.1. Structure of barley hull

The grain of barley is covered by a tough outer layer, known as husk or hull. The hull connected to the barley kernel is very difficult to detach by threshing as occurs with the hull-less barley available. Dendy and Dobraszczyk (2001) have shown that the hull contains two bracts: lemma and palea. The lemma is attached to the dorsal side of the grain while the palea is attached to the ventral side (Hough, 1991).

Barley grain is considered to be of good quality if it is bright in appearance and the hull color is buffed (Broderick and Vogel, 1977). Thus, if there is any environmental, climatic or disease damage, the grain would appear discolored (Fox et al., 2001). As shown in Fig. 2.1, a sound and ripe barley grain has three layers: the outer epidermis, fibers and spongy parenchyma

(Briggs, 1998; Olkku et al., 2005). Hull, highly lignified due to the silicified epidermis (Briggs, 1998), provides a physical barrier for micro-organisms initial attack to the barley grain (Agbagla-Dohnani et al., 2003). The epidermis and fiber layers are built with thick-walled cells, which act as a protective barrier, while the spongy parenchyma layer is made up of thin-walled cells (Olkku et al., 2005). Olkku et al. (2005) reported that this thin layer of the hull has great resistance towards physical and chemical damages. A cementing material which is secreted by the epidermis cells during the grain development is considered helpful in fusing the hull of the barley to the pericarp (Olkku et al., 2005). Thus, the specific structure and composition of the hull provide barley with an abrasive surface. The different varieties of barley have different hull thickness and resistance property (Olkku et al., 2005).

2.3.2. Variation in barley hull content

Barley grain has variable hull content that usually ranges from 7 to 25% on the dry matter basis. The average hull content in most barley varieties is 13% (Evers et al., 1999). There has been many factors reported, which affects the barley hull content in the different barley grains, such as variety, environment, agronomic practices and grain size.

(a) Variety: Barley hull content of different varieties changes on the basis of the genetics, which is associated with a region on chromosome 2H (Collins et al., 1998). For instance, the hull-less barley variety contains hull during its life-cycle, but it is loosely attached to the grain so it comes off easily from the grain during threshing. Broderick and Vogel (1977) and Evers et al. (1999) showed that the two row barley variety has less hull content than the six-row

barley. Thus, the variety effects on the hull content were complicated due to its relation to the plumpness, grain size and weight of barley hull.

(b) Growing environment: The change in latitude caused by the geographical differences may create a varied climate and weather conditions, which affects barley growing conditions. The growing environment can cause a change in the barley grain composition and directly or indirectly change the hull content. Evers et al. (2005) reported that varieties grown in the low latitude (equator regions) have high hull content than varieties grown in high altitude (earth's poles). This may be due to long day patterns, low temperature, less disease and predation stress, which reduces the plant cell wall content and the hull content (Van Soest, 1994). The grain filling development has been affected by the longer day-light, reducing the hull content (Slafer et al., 2002). Many studies have also reported that the high temperature and drought during the grain growing period hinders the starch accumulation and reduces the barley grain weight and size, which consequently increases the hull content of the grain (Roxana and Nicolas, 1996; Voltas et al., 1999; Passarella et al., 2002).

(c) Agronomic practices: Grove et al. (2003) have shown that the early-planted barley grain contains more hull than the later-planted barley grain. The early planted barley grain has more fibre (neutral and acid detergent) because of the use of excessive level of N-fertilizer, which affected the barley hull content (Erekul et al., 2007).

(d) Grain size: The physical and morphological aspects of barley grain also determine the hull content. Plumper barley grains have low hull content. (Du and Yu, 2012).

2.3.3. Chemical composition of barley hull

In general, Briggs (1998) and Olkku et al. (2005) reported that barley hull is an extremely fibrous material and is mainly composed of hemicellulose, cellulose, lignin, ash and protein (Table 2.1).

Grove et al. (2003) and Moore and Jung (2001) reported that variation in the content of the fiber is due to different barley hull varieties and different agronomic practices. Due to the contribution of the barley hull to the total fiber content in the barley grain, it becomes difficult for humans, and other monogastric animals, such as rats, pigs, and poultry to digest the fibrous hull due to fewer amounts of fibrolytic enzymes.

Ash, mainly silica (SiO_2), is also found in barley hull (5.1%) and in barley grain (1.5% in the peeled grain) (Kulp and Ponte, 2000; Olkku et al., 2005). The high amount of silica in the hull contributes to its strength, rigidity and integrity. Thus, the plant growth and grain development is improved resist abiotic (e.g. dry soil) and biotic (e.g. diseases) (Liang et al., 2003). Barley hull also contains significant amounts of phenolics. Lignin is considered a typical complex phenolic compound which restricts the digestion of the plant cell wall by animals (Priest and Stewart 2006). Also, free phenolic acids, such as ferulic acid and *p*-coumaric acid, are present in the barely hull (Slafer et al., 2002; Priest and Stewart 2006). The presence of these free phenolic acids defends barley grain from micro-organism attack due to their antioxidative properties and antibacterial functions.

The intricate cross-linkages between lignin and cell-wall polysaccharides with phenolic acids provide the inhibitory factors for the degradation of plant cell wall in the rumen.

Table 2.1 Chemical composition of barley hull (adapted from Olkku et al., 2005)

Component	Content (%)
Cellulose	26.5 - 28.8
Hemicellulose	32.4 - 33.7
Lignin	14.7-22.9
Ash	5.1 - 5.2
Protein	2.1 - 3.0
Others	6.7 - 8.0

*variety not mentioned

2.3.3.1. *Carbohydrates in barley hull*

Barley hull, an agricultural by-product with high carbohydrates content, can be a source for feed supplements as well as for the production of glucose or ethanol (Moldes et al., 2002). Due to the presence of cellulose, hemicellulose and lignin, barley hull is also known as a lignocellulosic material. Höije et al. (2005) reported the presence of 23% cellulose, 32.7% non-cellulosic polysaccharides, mainly arabinoxylan (hemicelluloses), and 21.4% lignin in the Cindy or waxy barley hull variety. Moreover, Garrote et al. (2004) reported protein, uronic acid and acetyl groups in the barley hull. Currently, barley hull application is limited to the animal feed or simply as a landfill. But, other potential applications of barley hull have also been suggested (e.g. as a food additive due to its high content of protein and fiber). Barley hull can also be utilized as a substrate for the cultivation of mushrooms and actinobacteria, and as a source of value-added products, such as ferulic acid, *p*-coumaric acid, xylose and arabinose (Mussatto et al., 2006; Mussatto et al., 2007).

Barley hull is considered the most abundant source of hemicellulose polymer, known as xylan. Xylan can be further hydrolysed into xylose and

arabinose (Fig 2.2) and then fermented to produce xylitol (a low caloric sweetener with anticarcinogenic properties). Xylitol can be used as a substitute of sugar for diabetic patients (Parajó et al., 1997; Cruz et al., 2007). However, due to low digestibility of barley hull, its utilization as a feed supplement is limited. The combustion of barley hull is also difficult and not practical due to its high ash content, which lead to the deposition of minerals in the boilers (Cruz et al., 2007; Garrote et al., 2008). Additionally, the transportation of barley hull to the disposal areas may be very expensive due to its low density (Mahmudi et al., 2005; Searcyl et al., 2007; Garrote et al., 2008). Due to these problems, the current interest of barley hull remains for the use in the saccharification and fermentation processes.

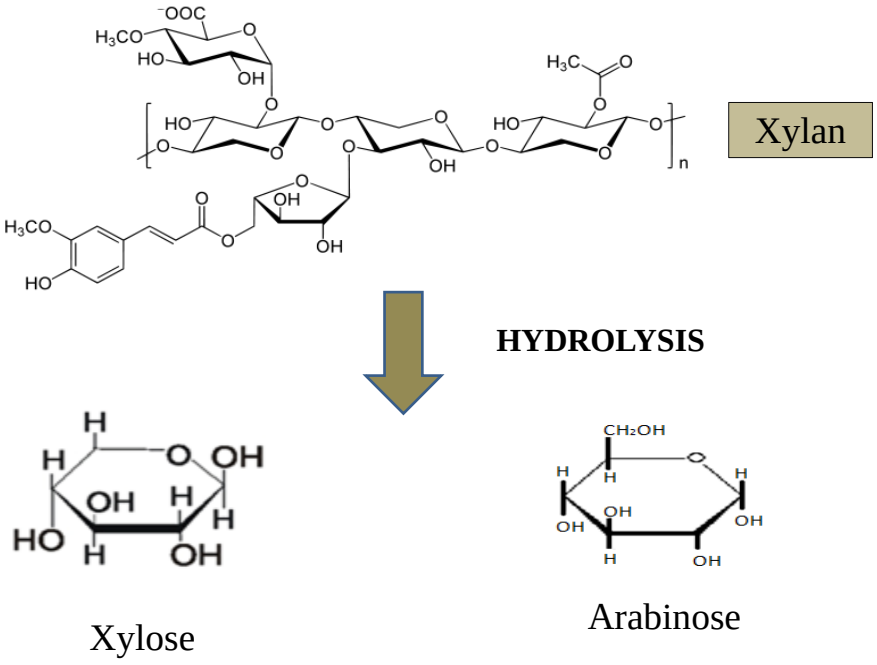


Figure 2.2 Hydrolysis of xyaln to xylose and arabinose (Adapted from Held 2012)

Kim et al. (2008) used aqueous ammonia at 30-75°C for 12 h to 11 weeks for the pre-treatment of barley hull, followed by the enzymatic saccharification for the bioconversion to fuel ethanol. However, phenolic compounds present in barley hull had inhibitory effect on the bacterial growth, requiring to be removed before the preparation of the fermentation media (Schwald et al., 1988). The utilization of phenolics compounds as antioxidants is discussed further in the next section.

2.3.3.2 Phenolics in barley hull

Garrote et al. (2008) quantified total phenolics as 33-36mg/g of barley hull. Earlier studies by Garrote et al. (2004) and Garrote et al. (2008) showed that non-isothermal treatment of barley hull in aqueous media degraded the hemicellulose fraction and selectively released sugar oligomers, monosaccharides, sugar degradation products (such as furfural, hydroxymethyl furfural, acetic acid) and phenolic compounds. Garrote et al. (2008) reacted barley hull with water at 185-260°C (autohydrolysis). The autohydrolysis process followed by the depolymerization of the lignin fraction (extraction of the liquor using ethyl acetate 1:3 v/v) resulted in benzoic acid and cinnamic acid as the major phenolic compounds in barley hull. On the other hand, Cruz et al. (2007) detected ferulic acid (2.5%) and *p*-coumaric acid (3%) as the major phenolic compounds in barley hull after the delignification of barley husk with 3% sulfuric acid followed by the treatment with 6.5% NaOH at 130°C for 60 min. The quantities of these two acids varied in the ethyl acetate solvent maintained at different pH conditions. The acidic pH (pH of 3) showed three times more ethyl acetate soluble phenolic compounds (99.9 mg/g) than the solvent maintained at pH 12.8 (31.3 mg/g).

The presence of high amounts of ferulic acid (43%) and *p*-coumaric acid (9%) in Brewer's spent barley grain, mainly formed by barley hull, were also reported by Bartolomé et al. (1997, 2003).

From decades, it is known that *p*-coumaric acid (trans-4-hydroxycinnamic acid) is the dominant phenolic in barley hull, forming linkages with lignin (Higuchi et al., 1967). Chemically, *p*-coumaric acid and ferulic acid (Fig 2.3) consist of a phenolic nucleus and an extended side chain, which readily generates stable phenoxyl radical that can scavange free radicals and prevent oxidative stress (Graf, 1992).

(a) (b)

Figure 2.3 Chemical structure of: (a) *p*-coumaric acid, and (b) ferulic acid (Adapted from Sutherland et al., 1983).

The interest towards these phenolic acids is due to their strong free radical scavenging capacity and chemoprotective effects (Mussatto et al., 2007). Abdel-Wahab (2003) suggested the use of *p*-coumaric acid for cancer due to its protection against doxorubicin-induced oxidative stress. Biological effects, which include inhibiton of LDL oxidation (Zang et al., 2000), reduction to oxidative damage in DNA (Guglielmi et al., 2003) and platelet aggredation inhibition (Luceri et al., 2007) were reported for the *p*-coumaric acid.

Ferulic acid, on the other hand, can be absorbed, metabolized and distributed and then excreted as a derivative of phenyl propionic acid, hydrocrylic acid and glycine conjugates (Srinivasan et al., 2007). Ferulic acid also proved to be beneficial in prevention of problems linked to oxidative stress which includes cancer (Chang et al., 2006), inflammatory diseases (Murakami et al., 2002), and Alzheimer's diseases (Jin et al., 2005). In China, sodium ferulate (a salt of ferulic acid) has been used against cardiovascular and cerebrovascular diseases (Wang and Ou-yang, 2005). Due to the antioxidant activity, free scavenging activity, chelation of active metal ions and modulation of gene expression, phenolics show pharmacological effects (Soobrattee et al., 2005). Therefore, research to establish an effective extraction process to obtain these compounds with high purity has been carried out for a long time.

2.4. Extraction methods

2.4.1. Conventional extraction

The utilization of carbohydrates and phenolics for the production of dietary supplements, nutraceuticals, food ingredients, pharmaceuticals, and cosmetic products depends on the extraction of these compounds from plant matrices. Solvent extraction is the most common extraction method that has long been used for the extraction of bioactives due to its ease of use. However, the yield of the extraction depends on the type of solvent, time, temperature, sample to solvent ratio, and the physico-chemical properties of the compounds. The chemical nature of the sample and polarity of the solvent are factors affecting the solubility of the compounds (Dai and Mumper, 2010). Different plant materials contain phenolics, which vary from simple (e.g.

phenolic acids, and anthocyanins) to more polymerized compounds (e.g. tannins). Moreover, phenolics are associated with other plant compounds, such as carbohydrates and proteins. Therefore, there is no common extraction method for the complete removal of phenolics from plant materials. Thus, depending on the solvent(s) used for the extraction, a mixture of phenolics with non-phenolic comounds, such as sugars and organic acids, can be extracted from different plant matrices (Dai and Mumper, 2010). Different solvents, such as methanol, ethanol, ethyl acetate, acetone, and their mixtures have been already employed for the extraction of phenolics, often used with varied concentrations of water. The selection of the solvent as well as its concentration is very important as it affects the amount and rate of phenolics extracted (Xu and Chang, 2007). The effectiveness of using methanol has been repoted for the extraction of low molecular weight polyphenols, such as anthocyanins, while aqueous acetone has been commonly employed for the extraction of high molecular weight flavanols (Metivier et al., 1968; Labarbe et al., 1999; Guyot et al., 2001; Prior et al., 2001). Ethanol is another effective solvent for the extraction of polyphenols (Shi et al., 2005). In addition, an acidified organic solvent (most commonly methanol or ethanol) is helpful for the extraction of polyphenolics from plant materials (Shi et al., 2005). The best extraction yield was obtained with the addition of weak organic acids, such as formic acid, acetic acid, citric acid, tartaric acid or low concentrations of strong acids, such as 0.5-3% of trifluoroacetic acid and < 1% of hydrochloric acid (Jackma, 1987; Revilla et al., 1998; Nicoué et al., 2007). The high acidic nature of the solvent system is beneficial to denature the cell membranes, simultaneously dissolving the polyphenolics. However, some detrimental

effects of excessive acid addition, such as degradation of labile sugars, may also be observed. To avoid these detrimental effects, other conventional method such as maceration (soaking) is also considered for the extraction of phenolics from plant matrices.

Solvent extraction is a process designed to remove soluble components, such as phenolics and carbohydrates, by diffusion of the solvent through the matrix. Solvent extraction mainly consists of two stages:

(a) **Initial stage** - Swelling of the matrix due to the desorption of the solvent in the matrix. The osmotic forces, which are caused by the capillarity and solvation of the ions in the cells, are the main factors for the sorption;

(b) **Diffusion stage** - Diffusion occurs within the matrix and through the outer layers that surround the targeted compounds (Giergielewicz-możajska et al., 2001).

The extraction time and temperature are important factors, influencing the recovery of phenolics (Robards, 2003). The increase of the extraction temperature can be beneficial to increase compounds solubility. Other factors, such as viscosity and surface tension of the solvent, are decreased at high temperatures, which allow the solvent to penetrate the matrix, improving the extraction rate. However, the conventional extraction method, such as maceration, has shown to be less efficient and time consuming. Thus, a number of alternative methods have been developed, such as supercritical fluid (SCF) extraction and subcritical fluid (sCF) extraction also known as pressurized fluid extraction (PFE) for the removal of phenolics from plant matrices.

2.4.2. Pressurized solvents

The use of pressurized solvents at elevated temperatures (more than 100°C in the case of water) and pressures increases the extraction rate by changing solvent density, diffusivity, viscosity, and dielectric constant (Kim et al., 2009). There are two pressurized fluid extraction systems:

(a) Supercritical fluid extraction (SFE): this method mainly uses CO_2 and water.

(b) Subcritical fluid (sCF) extraction: This is also known as accelerated solvent extraction (ASE), or pressurized fluid extraction (PFE). When water is used as a solvent, it is known as pressurized hot water extraction (PHWE) or pressurized low polarity water (PLPW) extraction, or superheated water extraction, or subcritical water (SCW) extraction.

Supercritical fluid extraction utilizes mainly CO_2, although other solvents, such as water and ethylene can also be used above its critical point, with a density similar to a fluid while the viscosity and diffusivity are similar to a gas. On the other hand, sCF extraction utilizes solvents, such as solutions of hexane, methanol or ethanol together or separately at different concentrations, above their boiling point under high enough pressure. SCW extraction is a special case of sCF extraction, where water is used as a solvent. Water is a polar solvent, but with the increase in temperature from 25 to 200°C under pressure, its dielectric constant decreases from 79 to 35, which is similar to the dielectric constant of ethanol (24) or methanol (33) (Cacace and Mazza, 2006; Saldaña et al., 2012). The three methods are further discussed in detail in the next section.

2.4.2.1. Supercritical fluid extraction

The extensive use of supercritical fluid technology for the extraction of phenolics from different matrices has already been reported mainly with supercritical CO_2. The most common example of scale up using SFE is the decaffeination of coffee beans (Zosel, 1981) and the extraction of bitterness and antimicrobial elements from hops for beer production (The Naked Scientists interviews, 2007). SFE is a process that uses a fluid above its critical pressure and temperature (Fig. 2.4). A supercritical fluid has properties of both a gas and a liquid (Palma and Taylor, 1999; Saldaña et al., 2002). Due to supercritical fluids unique physico-chemical properties (Table 2.2), they are considered advantageous over traditional solvents (Anklam et al., 1998; Saldaña et al., 2002).

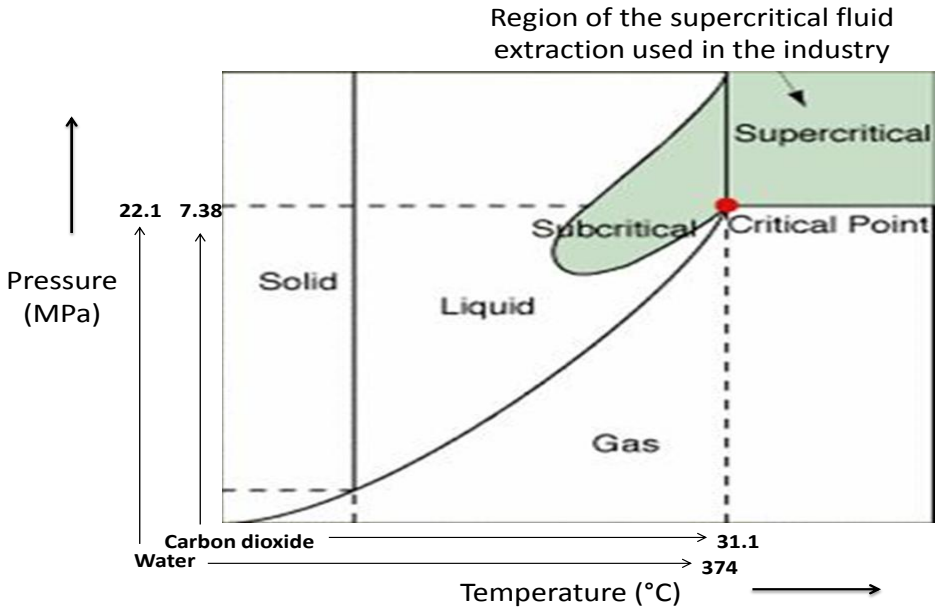


Figure 2.4 Phase diagram of water and CO_2 (Adapted from Herrero et al., 2006)

Studies reported the use of supercritical CO_2 for the extraction of antioxidants, and lipids, due to its relatively low critical temperature of 31.1°C

and critical pressure of 7.4MPa (CRC, 2008). In addition, the use of CO_2 can be beneficial as it is considered food grade and is available at a relatively low cost with high purity.

Table 2.2 Physico-chemical properties of gases, liquids and supercritical fluids (Adapted from Herrero et al., 2006)

Fluid state	Density (kg/m^3)	Diffusivity (mm^2/s)	Viscosity (µPa.s)
Gas (P = 1 atm; T= 21°C)	1	1-10	10
Liquid (P =1 atm; T = 15-30°C)	1000	0.001	500-1000
Supercritical (Pc = critical pressure; Tc = critical temperature	100-1000	0.01-0.1	50-100

Due to the low viscosity and high diffusivity of supercritical fluids, they have better transport properties than liquids. Thus, supercritical fluids can diffuse easily through plant matrices. The density of supercritical fluids can be modified by changing the temperature and pressure of the fluid. The solvation power of these fluids depends on the density, diffusion and viscosity. In addition, the surface tension of the supercritical fluid is relatively low.

Various parameters can be controlled during the SC-CO_2 treatment, such as the solvent:feed ratio, particle size of the feed, co-solvent concentration, temperature, pressure, time and flow rate (Reverchon and De Marco, 2006). Due to the high percentage of phenolics in grapes, apples, olives, and green tea, SC-CO_2 extraction has been used to remove phenolics. The Leatherhead Food Research (2009) has estimated that the current market for phenolics ranges to approximately $200 million. Table 2.3 shows the

optimal conditions for the extraction of phenolics from various plant sources using SC-CO_2.

Table 2.3 SC-CO_2 extraction of phenolics from plant by-products (Adapted from Wijngaard et al., 2012)

Source	Extraction condition	Total phenolics (Yield)	Reference
Apple and peach pomace	52-58°C, 50-57MPa, 30% ethanol, 40 min	250 mg/g (25%)	Adil et al. (2007)
Grape skin	45-46°C, 15.7-16.2MPa, and 6-7% ethanol	125 mg/g (12.5%)	Ghafoor et al. (2010)
Orange pomace	40°C, 30MPa, 2% ethanol	36 mg/g (3.6%)	Benelli et al. (2010)
Guava seed	60°C, 10MPa, 10% ethanol	1.53 mg/g (0.1%)	Castro-Vargas et al. (2010)
Pistachio hull	45°C, 35.5MPa, 15 min, 15% methanol	78.1 mg/g (7.8%)	Goli et al. (2005).
Olive leaves	100°C, 33MPa, 10% methanol	45%	Le Floch et al. (1998)

A number of polyphenolics have been removed with SC-CO_2 extraction, such as resveratol from grape by-products; however, the efficiency of the extraction was low measured as total phenolic content (Wijngaard et al., 2012). Due to the high polarity of polyphenolic compounds, a co-solvent (e.g. ethanol) needs to be added to the SC-CO_2 system. When ethanol is added as a co-solvent to the SC-CO_2, temperature and pressure need to be increased to maintain the mixture above its supercritical conditions. The addition of a co-solvent increases the solubility of phenolics in the supercritical solvent. Li et al. (2010) reported that usually ethanol is added at 10-20%, but this percentage of ethanol can also increase as high as 60%.

Polyphenols can undergo various molecular modifications, mainly hydroxylation, glycosylation, and polymerisation due to enzymatic catalysis (Wijngaard et al., 2012). Pinelo et al. (2006) reported that the extraction of monomeric polyphenols, such as catechins and phenolic acids, increased with

the use of SC-CO_2 as compared to the solid-liquid extraction using 96% aqueous ethanol. In addition, the use of 8% methanol has been shown effective for 30% extraction of catechin, epicatechin and gallic acid using SC-CO_2. But, the use of methanol restricts its application as a food ingredient due to heath regulations. Also, yields of phenolics using this technology are still low even with the use of a modifier. Therefore, other techniques, such as subcritical fluid extraction has been studied.

2.4.2.2. Subcritical fluid (sCF) extraction

SCW is a type of sCF technique based on the use of pure water as the solvent maintained at a temperature between 100 and 374°C (Fig. 2.4) and at a pressure, which is sufficient enough to keep the water to its liquid state. Due to the uniqueness of water as a solvent with high hydrogen bond structure, it has a high boiling point, high polarity and high dielectric constant at high temperature. The physico-chemical properties changes dramatically as a function of temperature (Hawthorne et al., 1994). For instance, there is a steady decrease in the permittivity, viscosity and surface tension but increase in the diffusivity. The use of elevated temperature decreases the dielectric constant (ε) to 27 at 250°C and 50 bar from a value of 80 when the temperature is maintained at 25°C. Thermodynamic calculations also show an increase of the ion product from 10^{-14} for liquid water (T = 25°C, P = 0.1MPa) to around10^{-11} in near critical conditions (T = 320°C, P = 25MPa) (Park and Park, 2002). Thus, under the subcritical conditions, water behaves as an organic solvent, such as methanol and ethanol, which can further dissolve a wide range of low polar solutes (King, 2000). Thus, the temperature change can be beneficial to tune the properties of the water.

Benthin et al. (1999) were the first to conduct a study on the feasibility of using subcritical fluids to extract diathrone, escin, and silybin from medicinal herbs after its introduction in 1990's. The combined use of pressure ranging from 3 to 20 MPa and temperatures of 40 to 250°C provides a rapid extraction with the advantage of small amounts of solvent use (e.g. time of extraction is less than 20 min, with 10-50mL of solvent) as compared to the traditional solid-liquid extraction, which requires 10 to 48 hr and with 200mL of solvent (Mendiola et al., 2007; Singh and Saldaña, 2011). Due to interest in the extraction of compounds from plant and herb sources, as well as due to the tendency of choosing a more green approach, sCF extraction is gaining a lot of attention. In addition, the equipment set-up for the sCF also provides the protection of oxygen and light sensitive compounds, such as phenolics and essential oils (Mustafa and Turner, 2011).

The high temperature during the sCF extraction increases the capacity of the solvent to solubilise the solutes, increasing the diffusion, disrupting of the solute-matrix bonds, and decreasing the viscosity and surface tension of the solvent (Ramos et al., 2002; Richter et al., 1996). The pressure is usually maintained in the range of 4 to 20 MPa, which ensures the solvent is maintained in a liquid state (Ramos et al., 2002). Thus, the solvent can drive into the pores of the matrix, enhancing the solubility of compunds (Ramos et al., 2002; Mustafa and Turner, 2011). However, due to the small effect of pressure during the extraction (Mustafa and Turner, 2011), most researchers use a constant pressure ranging from 4 to 20MPa (Singh and Saldaña, 2011).

A subcritical fluid is suitable for a wide range of solutes usually ranging from polar to non-polar compounds. Originally, the use of subcritical

fluids focused on the isolation of organic contaminants from environmental samples, such as soil, sediments and sewage sludge (Richter et al., 1996; Ramos et al., 2002; Smith, 2002; Mendiola et al., 2007). However, currently, this technique is applied in food and biological samples using different organic solvents under subcritical conditions. Instead of using an organic solvent, pure water is used for the extraction, this technique is known as subcritical water extraction.

The basic set-up of a subcritical fluid extraction is shown in Fig. 2.5. This system mainly consists of a stainless steel extraction vessel, which holds the sample; the temperature and pressure are controlled using electronically controlled heaters, pumps and a back pressure regulator to maintain the pressure constant. The final liquid extract is passed through a cooling system and then is collected in a vial (Saldaña et al., 2012).

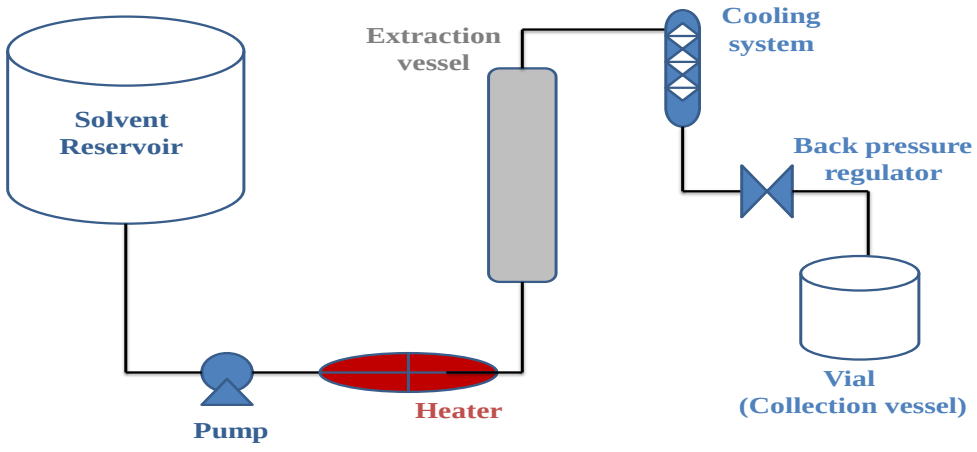


Figure 2.5 Schematic diagram of a subcritical fluid extraction system (Adapted from Singh and Saldaña, 2011)

The sCF extraction can be carried out in two modes: static mode and dynamic mode. In the static mode, the sample is first placed inside the

extraction vessel, which is then filled with the organic solvent and the vessel is pressurized. After heating the extractor to the required temperature, static extraction is carried out for approximately 5 - 20 min (Teo et al., 2010). The valve is then opened after the extraction time, and the solvent is allowed to flow to the collection vessel. For both modes, it is recommended to grind the sample before packing to improve diffusion and increase surface area. Finally, the extraction vessel is inserted at both ends with filters and glass wool plugs to prevent blocking of the tubing by small particles (Singh and Saldaña, 2011).

In the static mode, the extraction efficiency strongly depends on the partitioning of the target compound in the phases presented and solubility of the solutes. Thus, highly concentrated samples or low solubility compounds may show incomplete extraction due to the limited volume of the solvent (Teo et al., 2010). However, in the dynamic mode, the solvent (water in most applications) is continuously pumped through the extraction vessel at a constant flow rate.

During the sCF extraction, the polarity of the solvent should be close to that of the target compound(s). In cases, when solutes have a wide range of polarities, a mixture of low and high polar solvents is used instead of a single solvent. Alternatively, the extraction can also be carried out in two steps: one with a non-polar solvent and followed by the use of a polar solvent (Waksmundzka-Hajnos et al., 2004; Klejdus et al., 2004). The increase in temperature increases the efficiency of the sCF extraction as it increases the sample wetting, better penetration of the solvent and high diffusion and desorption rates of the solutes from the matrix to the solvent. This method is

therefore recommended as an extraction process if there are no limitations associated with the thermolabile solutes or matrices.

2.4.3. Parameters affecting extraction

The main parameters that influence the selectively and efficiency of SCW extraction include temperature, pressure, time, flow rate and co-solvent. The geometry and the flow direction of the extraction vessel had little impact on the recovery of the solutes from the sample matrix (Teo et al., 2010). Due to the high temperature, the other factors, such as sample matrix, has little effect on the recovery of non-polar solutes (Kronholm et al., 2003).

(a) **Temperature:** Temperature is considered the most important parameter. The temperature change affects the extraction rate, efficiency and selectivity. The ionic, hydrogen bonding and dipole-dipole interactions between the water molecules decrease with the increase in the temperature and thus lowers the dielectric constant (Yang et al., 1998). Increase in temperature disrupts the solute-matrix interactions and increase the solubility of the solutes.

The change in the viscosity of the solvent with temperature is observed from room temperatures to 100°C, enabling better penetration through the matrix. However, the decrease of surface tension due to the increase in temperature can allow the water to wet the sample matrix properly. These factors along with the improvement of the mass transfer that enhances recoveries of solutes using SCW are promising. For the extraction of polar solutes, relatively low temperatures (100-150°C) is recommended, whereas for moderately and low-polar solutes temperatures of 200-300°C are preferred (Kara et al., 2006).

The temperature is a measure of the kinetic energy of the system, thus an increase in temperature implies higher kinetic energy of the molecules and therefore more collisions per unit time. In theory, very high extraction temperatures are used for the extraction of non-polar analytes, but due to the instrumental challenges, such as corrosion and leakage, temperatures of more than 300°C are not recommended (Katritzky and Llin, 1995). In addition, compunds can degrade at high temperatures, decreasing their yield (Windal et al., 2000).

(b) **Pressure:** The most important advantage of using pressure is that the solvent temperature can be maintained above its boiling point, while the solvent is in the liquid state. The use of elevated pressures at high temperatures allows the solvent to pass through the matrix pore and extract the solutes. The force provided by the pressure results in the disruption of the matrix, which enhances the mass transfer of the solutes from the matrix to the solvent. However, the effect of pressure on the recovery of most solutes has been found to be negligible as long as the physical state of the water is not changed (Chienthavorn and Su-in, 2006).

(c) **Flow rate:** Flow rate is another factor, which affects the recovery of solutes and needs to be determined.

(d) **Modifiers and additives:** The addition of organic or inorganic modifiers and additives may enhance the solubility of the solutes in water. At elevated temperatures, they may also alter the physico-chemical properties of water. For instance, the subcritical water extraction containing 5% ethanol was reported to enhance the anthocyanin recovery from red cabbage (Kiathevest et al., 2009).

2.4.4. Comparison of SCW extraction with other extraction methods

There are benefits and advantages associated with the use of subcritical water in comparison with other extraction methods (Table 2.4). No harmful organic solvents are required for the SCW extraction. Hence, it can be used in food products. Since the selectivity of the solute is high, the SCW is considered to be a very effective method of extraction.

Table 2.4 Comparison of different extraction techniques

	Extraction Techniques		
	Solid-liquid	**SC-CO_2**	**SCW**
Typical extraction Time	4 to 48 hr	30 to 90 min	5 to 30 min
Typical solvent used	Acetone-hexane, dichloroethane, dichloromethane, toluene, methanol	SC-CO_2/SC-CO_2 + modifier	Water
Solvent consumption (mL)	100 to 300mL	8 to 90 mL	15 to 40 mL
Selectivity for analytes	Non-selective	Slightly selective	Selective
Benefits	Simple known procedure, easy, cheap equipment	No or little organic solvent required	No organic solvent needed
Disadvantages	Time consuming, more manual work, large consumption of solvent	Need modifiers with CO_2 to extract polar solutes	Not suitable for thermo-labile compounds

SCW is considered an emerging solvent for extraction due to the possibility of tuning the selectivity and efficiency of the extraction with the change of temperature (Ramos et al., 2002; Smith, 2002; Saldaña et al., 2012).

Superheated steam is a pre-treatment method which employs steam and makes the biomass more accessible for further processing such as fermentation (Neves et al., 2007). With this method, the biomass is rapidly heated (400°C) using pressure of 0.1 MPa for a specific period of time (Broll et al., 1999).

2.4.5. Application of SCW extraction

Recently, SCW has become a useful tool for the extraction of bioactives and nutritionally important compounds from different plant and food sources. In addition, the SCW can be used for the extraction of organic contaminants, such as pesticides, veterinary drugs and environmental chemicals from fruits, vegetables and meat product samples, which is useful in terms of food safety (Teo et al., 2009). The interest towards the use of SCW is mainly due to the fact that the system can be automated, with reduced time and less solvent consumption. Haiyee et al. (2009) showed that SCW extraction requires minimal sample preparation, such as homogenisation.

(a) Bioactives extraction from food and plant materials

Health Canada has proposed that a nutraceutical is a product isolated or purified from foods, which is generally sold as a medicinal and physiological benefit to protect against chronic diseases. Thus, different dietary fibers, phenolic compounds, polyunsaturated fatty acids, amino acids, proteins and minerals are considered nutraceuticals or bioactives. These compounds are beneficial due to their health promoting effects, such as reduction of the risk of heart diseases (Dauchet et al., 2006), cancer, hypertension (Zhang et al., 2009), and diabetes (Mignone et al., 2009). Thus, the extraction of bioactives from plant sources is important (Mustafa and Turner, 2011; Saldaña et al., 2012).

Table 2.5 shows the studies on the extraction of bioactive compounds from plant sources using SCW technology. The extraction efficiency depends on the type of compound extracted. Thus, the operating parameters for the extraction of individual compounds may vary. As shown in Table 2.5,

operating conditions for the extraction of anthocyanins, procyanidins, and flavanols differ as solubilities of these compounds vary. The solubility of the polyphenol in the solvent depends on the polarity, size and characteristics of the compound and the solvent.

Temperature also affects the extraction yield, as it enhances the mass transfer and extraction rates. The optimal temperature during SCW ranges between 100 to 250°C depending on the compound to be extracted and its matrix (Table 2.5). Garcia-Marino et al. (2006) found that the sum of the individual polyphenols from winery by-products also increased at an elevated high temperature (180°C). In addition, the extraction of individual phenolic acids, such as gallic, chlorogenic, caffeic, ferulic, and coumaric acids, increased with an increase of temperature from 120 to 200°C (Rangsriwong et al., 2009), while temperatures above 180°C can degrade phenolic acids (Singh and Saldaña, 2011). Therefore, temperature should be considered when working with thermo-labile bioactive compounds. In addition, Maillard reaction products may be produced using SCW at high temperatures (Monrad et al., 2010; Wijngaard and Brunton, 2009). Thus, temperature needs to be considered while designing an optimal SCW process.

The highest total sugar in defatted rice bran was obtained using SCW at 200°C (Hata et al., 2008). For the SCW extraction of catechins and proanthocyanidins of dried grape seeds, the results were similar to the conventional extraction with 75% methanol (Marino et al., 2006). Using SCW, different capsaicin of peppers was successfully extracted at 200°C but the higher temperatures decreased the yield (Barbero et al., 2006). In general, the anthocyanin extraction was carried out at a temperature of 120-160°C.

Table 2.5 Compounds extracted using SCW technology

Source	Target Compound	Operating Conditions	Yield (mg/g)	References
Agave americana	Total phenolics	150°C, 1MPa, 240 min	23.8	Ben Hamissa et al. (2012)
Bitter melon (Plant)	Phenolic compounds	150-200°C, 10MPa, 350 min	52.63	Budrat and Shotipruk,(2009)
Canola meal	Total phenolics	160°C, 6.89MPa, 30 min	15.66	Hassasroudsari et al. (2009)
Citrus unshiu peel	Flavanones	160°C, 10.1MPa, 10 min	83.7	Cheigh et al. (2012)
Coffee silverskin	Total sugar, reducing sugar, protein, total phenolics	25-270°C, 5.3MPa, 10 min	121, 52, 157, 36	Narita and Inouye (2012)
Mango leaves	Phenolic compounds	100°C, 4MPa, 180 min	17.74	Fernandez Ponce et al. (2012)
Olive leaves	5-hydroxymethylfurfural	200°C, 10.3MPa, 20 min	3.17	Herrero et al. (2012)
Onion skin	Flavonols	100-190°C, 9-13.1MPa, 15 min	16.29	Ko et al. (2011)
Pomegranate peel	Polyphenols	40-90°C, 10.2MPa, 5 min	264.3	Çam and Hışıl (2010)
Pomegranate seed residue	Phenolic compounds	100-220°C, 6MPa, 30 min	48.5	He et al. (2012)
Potato peel	Phenolic acids	180°C, 6MPa, 60 min	0.82	Singh and Saldaña (2011)
Rice bran	Carbohydrates, total phenolics	100-360°C, 6.2MPa, 10 min	215, 42	Pourali et al. (2010)
Winery waste	Total phenolics, flavonoids	140°C, 11.6MPa, 30 min	31.69, 15.28	Alikabarian et al. (2012)
Yellow Myrobalan (Plant)	Gallic acid, ellagic acid, corilagin	120-200°C, 4MPa, 150 min	15.47, 5.59, 6.11	Rangsriwonga et al. (2009)

Table 2.5 Continued				
Source	**Target Compound**	**Operating Conditions**	**Yield (mg/g)**	**References**
Aspen knotwood	Flavonoids	150°C, 22MPa, 35 min	38.82	Hartonen et al. (2007)
Defatted flax seed meal	Lignans, proteins and carbohydrates	130-190°C, 5.2MPa, 400 min	21, 225, 215	Ho et al. (2007)
Defatted rice bran	Total sugars, proteins	200°C, 5 min	0.3	Hata et al. (2008)
Defatted soyabean flakes	Isoflavones	110°C, 4.4MPa, 138 min	3.99	Chang et al. (2004)
Dried grape skin	Total phenolics	100-160°C, 40s	52.3	Ju and Howard (2005)
Flax or Linseed	Lignans	140°C, 5.2MPa, 400 min	9.39	Cacace and Mazza (2006)
Peppers	Capsaicin, dihydrocapsaicin	50-200°C and 10MPa	22.1, 27.7	Barbero et al. (2006)
Spirulina platensis (Microalgae)	Anti-oxidants	60-170°C, 10MPa, 3-15 min	9.80	Herrero et al. (2005)
Tea leaves	Catechin, epicatechin	100-200°C, 10MPa, 10 min	0.62, 3.31	Piñeiro et al. (2004)
Winery by-products	Polyphenols	150°C and 6-7MPa	19.3	García-Marino et al. (2006)
Winged yam (vegetable)	1,1-Diphenyl-2-picrylhydrazl	120°C, 2.17MPa, 180 min	71.15 %	Chen et al. (2004)

(b) Removal of contaminants from food

Chemical contaminants are compounds that can potentially harm the health of humans, wildlife and aquatic life. These contaminants include pesticides, veterinary drugs, and other chemicals, such as polychlorinated biphenyls (PCB's), polychlorinated aromatic hydrocarbons (PAH's) (Bogialli and Di Corcia, 2007). The extraction of these contaminants from food is usually associated with a long extraction time (18-24 h) and use of different clean-up procedures (Mendiola et al., 2007; Marazuela and Bogialli, 2009). Hence, due to the various advantages of the SCW discussed earlier, this green technology can be employed to extract various toxic compounds of food matrices. For example, sulfonamides, which are bacteriostatic compounds, routinely used as a veterinary medicine to treat a variety of bacterial and protozin diseases in poultry, may be carcinogenic and thus pose a health risk. SCW in the dynamic mode (1 mL/min of water at 160°C) effectively removed sulfonamides from meat samples (Marazuela and Bogialli, 2009). In addition, SCW is feasible technique to extract harmful pesticides and herbicides, added for fungal and pest control, which may remain in food and animal feed. For example, pesticides such as lindane, vinclozolin, quinalphos, procymidone etc. have been extracted from the skin of grapes with SCW at 120°C, 1 mL/min, and 40 min (Lüthje et al., 2005). In addition, herbicides, such as chloremquat and mepiquat in wheat flours and baby food samples were also extracted using a static extraction for 15 min at 120°C (Marchese et al., 2009). Hence, the SCW has gradually increased its application in the contaminant extraction area for food safety analysis.

In the environmental area the application of SCW to extract some of the polycyclic aromatic hydrocarbons (PAH`s) from soil under appropriate experimental conditions (50-250°C, 5 MPa and 1mL/min) was reported (Hawthrone et al., 1994). The solubility behavior of three PAH's, namely acenaphthene, anthracene, and pyrene, in SCW was studied at 50 - 300°C to understand the mechanisms of extraction of SCW (Andersson et al., 2005). It has been observed that the treatment of SCW at a temperature less than 250°C for 1 to 2 h was effective for the extraction of PAH's from soil samples. Thus, SCW can also aid in the soil/sediment remediation effort for the environmental monitoring and safety.

2.4.6. Future perspectives of SCW extraction

The likely future of this technology is towards scaling up of the process. The design of industrial scale equipment is usually preceded by laboratory and pilot scale systems to obtain sufficient preliminary data. The key factors of SCW extraction, such as temperature, pressure, flow rate or pH, are usually fixed to obtain desirable extraction efficiency (Pronyk and Mazza, 2009). Hence, with some modifications, SCW can be scaled-up to extract high volumes of desirable compounds from many solid or powdered samples, such as plant and food matrices. Thus, the SCW is a feasible green technology, which can be explored for various compounds targeting industrial applications, such as terpenes and phenolics.

2.5. Ionic Liquids

2.5.1. Introduction

Ionic liquids (ILs) are a group of new salts that are liquids at relatively low temperatures (below 100°C). As its name suggests, they are comprised of a collection of cations and anions of hydrophobic or hydrophilic nature. However, comparing with traditional molecular solvents, ILs have shown to have very interesting properties, such as low melting point, high thermal stability and negligible vapour pressure (Krossing et al., 2006).

ILs are also known as green solvents because they have low vapor pressures (Anderson et al., 2002). Depending on the choice of anion and cation combination, their physical and chemical properties, such as melting point, viscosity, hydrophobicity and hydrolysis stability can be adjusted accordingly (Huddleston et al., 2001). Therefore, optimal ILs can be designed for the development of more efficient processes and products by simply changing the structure of either the anion or the cation, or both. A better tuning of their properties is possible by varying the length and branching of the alkyl groups that are incorporated to the cation or anion.

Due to their beneficial properties, ILs have already been used as a solvent in various catalytic reactions (production of fine chemicals, pharmaceuticals, etc.) (Muldoon, 2001), separation processes (Roth, 2009; Zhang et al., 2004), synthesis (Welton, 1999; Wasserscheild and Welton, 2003), electrochemistry methods (Reddy, 2009; MacFarlane et al., 2007; Hapiost and Lagrost, 2007) and extractions (Huddleston, 1998; Zhao, 2005). Many ILs based on imidazolium salts have been utilized in various applications. But, due to their resistance to photodegradation (Stepnowski and

Zaleska, 2005) and low biodegradability (Gathergood and Scammells, 2002), ILs are considered a pollutant solvent and increase the production cost of the final product. Thus, these disadvantages led to the development of new classes of ILs that belonged to the hydroxyl ammonium family, providing the advantage of lower cost and simplicity of synthesis (Alvarez et al., 2010). There have been studies by Yuan et al. (2007), Alvarez et al. (2010) and Cota et al. (2007) for the synthesis of several ILs, such as 2-hydroxyethylammonium formate, *N*-methyl-2-hydroxyethylammonium acetate, 2-hydroxyethylammonium acetate, etc., belonging to the hydroxyl ethylammonium family. The various ILs from this family were synthesized by simply modifying the aliphatic chain of the organic acid and/or using secondary and tertiary hydroxylamines.

The structural study of the pure IL was performed using Nuclear Magnetic Resonance (NMR) experiments, which showed that due to the amphiphilic nature of the IL, the alkyl chains of the anion may combine to form a lamellar or micellar liquid-crystal phase. The highly hydrophobic alkyl chains of the IL anion are packed together in the lamellar/micellar phase. Thus, the polar carboxylate anion groups are exposed to the hydrophilic interlamellar/micellar space, which makes them group with the cation species and also to trace water present (Alvarez et al., 2010). In addition, it was observed that the self diffusion coefficient of the IL was increased with the increase of alkyl chain of the IL anion. The diffusion coefficient results in the ordered lamellar/micellar liquid-crystal phase due to the presence of alkyl chain in the anion species. Thus, it was suggested that larger the alkyl chain, the larger is the fluidity of the lamellar phase. The larger alkyl chain length of

the IL anion was also helpful in increasing the apparent viscosity and refractive index. After understanding the properties of the ILs, they can be applied in the solubilisation of carbohydrates or extraction of bioactive compounds from crops.

2.5.2. Advantages of ILs over conventional organic solvents

- ILs have the ability to dissolve many different organic, inorganic and organometallic materials (Welton et al., 1999).
- ILs are highly polar (Gorman et al., 2001)
- ILs consist of loosely coordinating bulky ions (Brennecke et al., 2001)
- ILs do not evaporate since they have very low vapor pressures (Yang et al., 2004)
- ILs are thermally stable, up to temperatures of about 150 to 300 ◦C (Yang et al., 2004)
- Most of ILs have a liquid phase up to 200°C, which enables wide kinetic control (Seddon et al., 1996).
- ILs have high thermal conductivity and a large electrochemical control (Lagrost et al., 2003).
- ILs are immiscible with many organic/inorganic solvents.
- ILs are non aqueous polar alternatives for various processes.
- The properties of ILs can be tuned for a specific application by varying the anion and cation combinations (Seddon et al., 1996).

2.5.3. Main properties of hydroxyl ethylammonium based ILs

Alvarez et al. (2010) studied the different properties of m-2-HEAA, such as density, speed of sound, refractive index, apparent viscosity, and self-

diffusion coefficient at 25°C. The density and speed of sound of pure m-2-HEAA was measured using a densimeter, while the apparent viscosity and refractive index were measured using a rheometer and a refractometer, respectively. The main properties of m-2-HEAA at 25°C are shown in Table 2.6.

Table 2.6 Properties of m-2-HEAA IL at 25°C (Adapted from Alvarez et al., 2010)

Property	**Calculated values**
Molecular weight, *M* (g/mol)	135.2
Density, ρ (g/cm^3)	1.1
Speed of sound, *u* (m/s)	1794.8
Refractive index, RI	1.4
Apparent viscosity, η (mPa.s)	106.1
Molecular radius, *r* (Å)	2.3
Melting point (°C)	-80

For this thesis, *N*-methyl-2-hydroxyethylammonium acetate (m-2-HEAA) was selected due to its easy synthesis, low cost, negligible toxicity, and easy degradability.

The phase equilibrium of ILs in SC-CO_2 has been studied extensively (Kim et al., 2012; Alvarez and Saldana, 2012; Manic et al., 2012; Iguchi et al., 2012; Planeta et al., 2012). However, there are no reports on the use of ILs in subcritical media.

Chapter 3: Materials and Methods

3.1. Materials

A new variety of hulled barley BT 584 was provided by Alberta Agriculture (Lacombe, AB, Canada). Barley grains were stored in a bag and kept at room temperature in a dry place until further needed.

Chemical reagents, such as sulphuric acid (97%, ACS reagent), ethanol (99.9%, HPLC grade), sodium hydroxide pellets (≥97%, ACS grade), Folin-Ciocalteau's phenol reagent (2M), gallic acid standard (99.9% purity) and D-(+)-glucose standard (99% purity) were purchased from Fisher Scientific Co. Ltd (Toronto, ON, Canada). Glass beads (~ 3mm) and glass wool were also purchased from Fisher Scientific Co. Ltd (Toronto, ON, Canada). The reagents used for the DPPH and FRAP antioxidant activity analysis, such as DPPH (99.99% purity), Fe_2SO_4 (98% purity), tripyridyltriazine and ferric chlorides were obtained from Sigma Aldrich (Oakville, ON, Canada). The m-2-HEAA ionic liquid was prepared using 2-(Methylamino)ethanol (99% purity) and acetic acid (99% purity) that were purchased from Sigma Aldrich (Oakville, ON, Canada).

3.2. Sample preparation

The hull was removed from the BT 584 barley using a dehuller (Buhler MLU 202 Flour mill, Markham, ON, Canada). The dehuller was equipped with break rolls (front and back) and reduction rolls. The hulls were then collected using an aspirator (model 3010-030, Udy Corp., Ft. Collins, CO, USA). The collected barley hulls were then milled to a desired particle size using a Retsch mill (ZM 200, Burlington, ON, Canada) and sieved to obtain

particles of 0.5 – 0.7 mm. The ground samples were vacuum packed in various moisture and oxygen barrier plastic containers, labelled and stored at -18°C until further use for the extraction experiments.

3.3. Methods

3.3.1. Proximate compositional analysis

Each proximate analysis described below was carried out in duplicates.

3.3.1.1.Moisture

Moisture content of hull was determined by a gravimetric method (AOAC, 2000). Approximately two grams of the barley hull was weighed using an analytical balance (Mettler Toledo, Mississauga, ON, Canada). Then, this sample was uniformly spread into pre-weighed and dried aluminium dishes (50 mm diameter x 23 mm deep). The dishes were then placed into a hot air oven (Model 655G, Fisher Scientific IsoTemp® oven, Toronto, ON, Canada) maintained at 105°C for 3 h. After drying, the dishes were partially covered with a lid and transferred into the desiccators for cooling. The weight of the dishes containing the dried sample was then recorded. The moisture content (%) was calculated using the following equation:

$$\% \text{ Moisture content} = 100 \times \left(\frac{A-B}{A}\right) \quad (3.1)$$

where, A = Weight (g) of the sample before drying, and

B = Weight (g) of the sample after drying

3.3.1.2.Ash

Ash content of the barley hull was determined by incineration of the sample at 550°C (AOAC, 2000). One gram of the barley hull was weighed in pre-weighed porcelain crucibles. The samples were incinerated overnight in a

muffle furnace (Model F-A1730, Thermolyne Corporation, Dubuque, IA, USA) at 550°C. The crucibles were then removed from the muffle furnace and cooled inside the desiccators. The crucibles were again weighed and the ash content was calculated according to the following formula:

$$\%\text{Ash content} = \frac{\text{Weight of ash (g) after incineration}}{\text{Weight of sample (g)}} \times 100 \quad (3.2)$$

3.3.1.3.Protein

Protein content was calculated by determining the nitrogen content using the Leco TruSpec nitrogen analyser (Leco instruments Ltd., Mississauga, ON, Canada). This method is considered a time and cost-efficient alternative to the Kjeldhal digestion method. Ground barley hull (approximately 0.16 g) was weighed into an aluminum foil cone and then pressed to form pellets. The encapsulated sample was then placed into the loading head. The system is then sealed and purged the sample to avoid any atmospheric gases that might have entered during the loading of the sample. The combustion of the sample was done inside a furnace at 950°C using pure oxygen. The reduced aliquot was measured by a thermal conductivity cell for nitrogen content (%). The calibration of the apparatus was done using a corn starch standard. Protein content was estimated using a conversion factor of 6.25 previously reported for barley (Temelli, 1997)

$$\%\text{ Protein content} = 6.25 \times Nitrogen\ content\ (\%) \quad (3.3)$$

3.3.1.4.Fat

The fat content of the barley hull was determined using a Goldfisch extraction unit (Labconco Co., Kansas, MO, USA), used by AOAC as a standard method for fat analysis. This method determines the weight of the fat

recovered after extracting it from the sample with a solvent. Two grams of the sample were weighed into the extraction thimbles ((25 mm I.D. x 80 mm length), Whattman International Ltd., Maidstone, England). The extraction thimbles were weighed and then approximately 40mL of petroleum ether was added to the sample in the thimble. A blank sample, containing 40mL of petroleum ether, was prepared and run through the entire extraction process. The extraction thimbles were then attached to the unit and the system was heated to a temperature of 60°C for 5 h. The extraction time allowed proper contact of the solute with the solvent, and thus enhanced the extraction of the fat from the sample. At the end of the extraction period, petroleum ether was evaporated from the beakers which were placed in an oven (Model 655G, Fisher Scientific IsoTemp® oven, Toronto, ON, Canada) at 110°C for 30 min to remove any residual moisture. The thimbles were then cooled in a dessicator and weighed. The fat content was calculated by the following equation:

$$\% \text{ Fat content} = \frac{\text{Weight of fat recovered (g)}}{\text{Weight of sample (g)}} \times 100 \qquad (3.4)$$

3.3.1.5.Carbohydrates

The total carbohydrate content of the barley hull for the proximate compositional analysis only was calculated by the difference of 100% minus the sum of the percentage of moisture, ash, protein and fat contents.

3.3.1.6.Pentosan content

The pentosan content of the hull was measured using the methodology described by Hashimoto et al. (1987) with some modifications. Pentosans are the non-cellulosic polysaccharides composed of arabinose and

xylose. Thus, the percentage of the pentosan can be considered as the hemicelluloses content of the sample.

For the pentosan analysis, 30 mg of ground barley hulls were reacted with 2mL of 2M HCl inside sealed test-tubes. The hydrolysis of the barley hull was performed inside the hot air oven set at 100°C for 2.5 h. After cooling of the test-tubes in a water bath, 2mL of 1M sodium carbonate (Na_2CO_3) solution and 2mL of the yeast solution (10mg/mL in 0.2M sodium phosphate buffer of pH 7.0) were added. One blank yeast sample containing a mixture of 2mL HCl, 2mL Na_2CO_3 and 2mL of yeast was also prepared. The test-tubes were then transferred in a water-bath maintained at 37°C and kept for 2 h to ferment the sample. The method was followed by vortexing 18mL of Milli-Q water with the mixtures. The solution was then centrifuged at 3500 rpm for 5 min. From this centrifuged sample, 1mL of the supernatant was collected in separate test-tubes. Then, 0.3mL orcinol solution (1% in ethanol) and 3mL $FeCl_3$ were added to each test-tube. After vortexing the test-tubes, the mixture was boiled in a water bath for 45 min, followed by cooling of the mixture. This mixture was then filtered through Whattman filter paper # 2. The absorbance of the filtered mixture at 670nm was then recorded. The standard curve was also prepared using xylose standard solution, and the slope of the curve was used for the calculation of pentosan. The following equation was used for calculating the percentage of pentosan in the sample:

$$\% \text{ Pentosan} = (\text{As} - \text{Ab}) \times (\text{m}) \times \left(\frac{1}{\text{W}}\right) \times 6 \times 0.88 \times 4 \times \frac{100}{1000} \qquad (3.5)$$

where, *As* = absorbance of the sample
Ab = absorbance of the blank
m = slope of the xylose standard curve

w = weight of the analyzed sample (mg)
0.88 = adjustment from free pentose to anhydro pentose (132/150)
100/1000 = conversion of micrograms to percentage
4 = dilution factor

3.3.1.7.Lignin Content

The lignin content of the barley hull was quantitatively determined using the TAPPI method (T 222, 2006). This method depends on the hydrolysis and solubilization of carbohydrates in the hull using 72% sulfuric acid. It is important to maintain the concentration of sulfuric acid to 72% because at concentrations below 65%, the hull is not completely hydrolysed, while concentrations higher that 80% lead to the precipitation of insoluble products from polysaccharides.

To estimate the amount of lignin in barley hull, the hull was first milled to a particle size of less than 1 mm of diameter using a Retsch mill (ZM 200, Burlington, ON, Canada). Then, 2 g of milled barley hull was hydrolyzed with 40mL of 72% sulfuric acid. The hydrolysis was carried out for 2 h in a water bath kept at 30°C. Then, the mixture was washed up with 400mL of Milli-Q water and transferred to a beaker. The volume of the mixture was increased to 1540mL by the addition of water to attain the concentration of the sulfuric acid of 4%. This mixture was then boiled for 4 h, maintaining the volume of the mixture at 1540mL. After boiling of the solution, the beaker was kept in an inclined position overnight to achieve the settling of the insoluble material, known as lignin (parameter A of equation 3.6). The supernatant was then decanted off and the insoluble material was filtered off using hot water. The collected insoluble material was load in a crucible and weighed. The crucible was then dried in a hot air oven at 105°C. The weight of

the lignin was recorded after cooling the crucible in a dessicator. Lignin (%) was obtained using the equation:

$$\% \text{ Lignin} = \frac{A \times 100}{B} \quad (3.6)$$

where, A = weight of lignin, g
B = oven-dried weight of sample, g

3.3.1.8.Cellulose content

The cellulose content of the barley hull was calculated by subtraction of the amount of pentosan content and the lignin content from the total carbohydrates of the sample.

3.3.1.9.Total phenolics

Total phenolics of the barley hull are reported as the sum of free and bound phenolics of the barley hull.

(a) Free phenolics

To obtain free phenolics, the methodology earlier described by Zhao et al. (2006) was used. Ground barley hull (10g) was extracted with 100mL of a mixture of ethanol:water (1:10 v/v) using a water bath at 65°C for 3 h. After centrifugation at 5000g for 10 min, the supernatant was removed and the obtained extract was collected and stored at -18°C until further analysis.

(b) Bound phenolics

As described by Bonoli et al. (2004), 5 g of the sample was digested with 20mL of 4M NaOH in a water bath at 65°C for 4 h to saponify the phenolics and carbohydrates bond. Then, the mixture was acidified at pH 2-3 with hydrochloric acid to avoid the formation of the bonds again and the solution was centrifuged at 4000 rpm for approximately 5 min. The

supernatant was then extracted two consecutive times with 100mL of petroleum ether using a separatory funnel. The extracts were stored for further analysis.

3.3.2. Extraction methods

Three different extraction methods were used during this study: solid-liquid extraction (with aqueous ethanol and with IL), subcritical fluid (sCF) extraction (with water, with aqueous ethanol and with aqueous IL), and supercritical CO_2 (SC-CO_2).

The use of aqueous IL (N-methyl-2-hydroxyethyl ammonium acetate, m-2-HEAA) under normal and subcritical conditions was studied for the first time for the extraction of total phenolics and total carbohydrates in this thesis.

3.3.2.1. Solid-liquid extraction

Solid-liquid extraction depends on the dissolution of one or more solutes from the solid matrix by a liquid solvent. In this study, the solid-liquid batch extraction was performed inside a tightly sealed volumetric flask, consisting of a magnetic stirrer to maintain a constant stirring throughout the process. The extraction was carried out using a water-bath which temperature was controlled by thermometers.

The extraction was performed using 0.25 g of ground barley hull and 45mL of a mixture of ethanol and water solution at different concentrations. The variables studied to optimize the extraction process were temperature, pH and aqueous ethanol concentration. The temperature was controlled using the heater in the water bath while the desired pH was maintained by the addition of 1% glacial acetic acid or 10% sodium hydroxide solution, depending on the acidic or basic pH conditions, respectively.

Response surface methodology (RSM) was used to determine the optimal conditions for solid-liquid extraction due to the effectiveness of the RSM in evaluating the effects and interactions of various factors of a particular response (Box and Wilson, 1951). The complete design consisted of 20 experiments, including 6 replications of the center point for the three independent variables (Table 3.1). Table 3.1 shows the theoretical values obtained with the software Design Expert v6.06 (Minneapolis, MN, USA) as well as the experimental values. The fitness of the model was determined by evaluating the Fisher test value (*F*-value) and the coefficient of multiple determinations (R^2) obtained by the analysis of variance (ANOVA). The central composite design used least square regression to fit the experimental data to a quadratic model.

Table 3.1 Response surface methodology for solid-liquid extraction**.**

Run	Theoretical conditions			Experimental conditions		
	Temperature (°C)	Ethanol (%)	pH	Temperature (°C)	Ethanol (%)	pH
1	60	0	8	60.00	0	8.00
2*	60	41	8	60.00	41.25	8.00
3	60	41	12	60.00	41.25	12.00
4	54	17	6	54.05	16.72	5.62
5	66	17	6	65.95	16.72	5.62
6	60	83	8	60.00	82.50	8.00
7	50	41	8	50.00	41.25	8.00
8*	60	41	8	60.00	41.25	8.00
9	54	17	10	54.05	16.72	10.38
10	60	41	4	60.00	41.25	4.00
11	54	66	6	54.05	65.78	5.62
12*	60	41	8	60.00	41.25	8.00
13	66	66	10	65.95	65.78	10.38
14*	60	41	8	60.00	41.25	8.00
15	70	41	8	70.00	41.25	8.00
16*	60	41	8	60.00	41.25	8.00
17	66	17	10	65.95	16.72	10.38
18	54	66	10	54.05	65.78	10.38
19*	60	41	8	60.00	41.25	8.00
20	66	66	6	65.95	65.78	5.62

* Center point (Temperature = 60°C, pH = 8 and ethanol concentration = 41%)

Each experiment was carried out for 3 h. This extraction time was calculated by monitoring the time required to obtain a constant refractive index of the extract while performing the extraction at the best conditions (70°C, 83% ethanol and pH of 12) of the factorial design (Table 3.1). The extract of each run was filtered using Whattman filter paper #4. The filtrates were collected in glass vials and kept in a freezer at -18°C until further analysis.

3.3.2.2. Subcritical fluid (sCF) extraction

The sCF extraction was performed using aqueous ethanol and aqueous IL solutions under subcritical conditions. The sCF extraction was carried out in a semi-batch type reactor (Figure 3.1), earlier described in detail by Singh and Saldaña (2011) and Saldaña et al. (2012). The extraction system consisted of an HPLC pump (Gilson 305, Villiers-le-Bel, Paris, France), a pre-heater, an extraction cell (Swagelok, Edmonton, AB, Canada) surrounded by heating brackets (TruTemp. Edmonton, AB, Canada), a pressure gauge (Swagelok, Edmonton, AB, Canada), a cooling system (Swagelok, Edmonton, AB, Canada) and a back pressure regulator (Tescom-26-1761-22, Elk river, MN, USA). The maximum pressure and temperature that can be achieved with this unit are 26 MPa and 315°C, respectively. All extractions were carried out using an extraction cell (1.3 cm internal diameter x 20.3 cm length), which was closed with inlet and outlet filters of 20µm. The sample (1 g) was mixed with glass beads of 2.3mm of diameter and then placed into the extraction cell. The inlet and outlet of the extraction vessel was filled with glass wool (2 mm thick) to avoid breaking the filters. The temperature of the system was

monitored by a thermocouple and the pressure was maintained constant with a back pressure regulator. The extracts were stored at -18°C for further analysis.

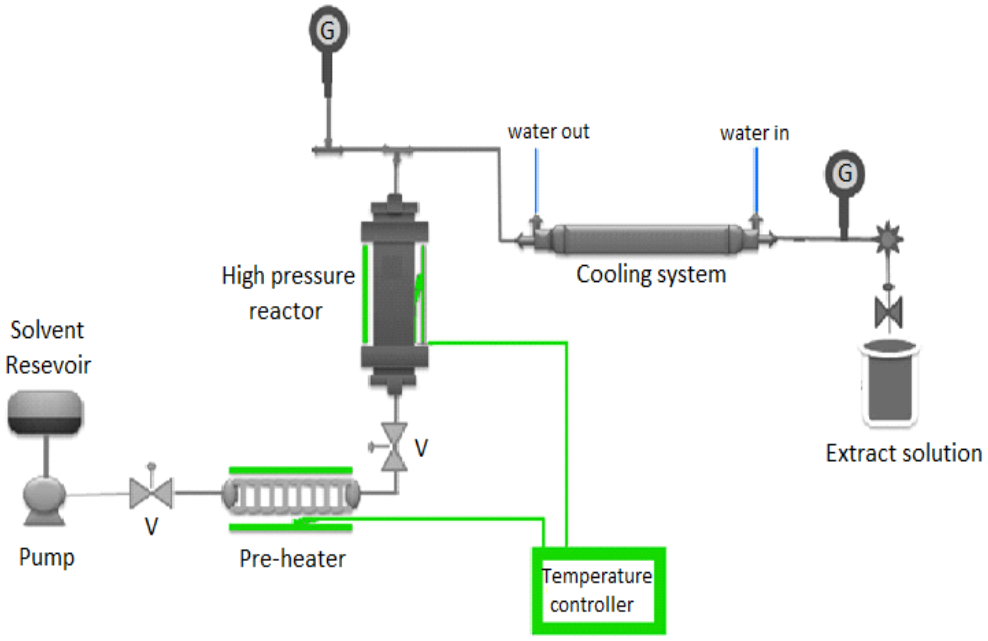


Figure 3.1 Subcritical fluid extraction system (G: Gauge; V: Valve) (Adapted from Saldaña et al., 2012)

3.3.2.2.1. Subcritical water+ethanol

The variables studied for the sCF extraction were temperature (°C), static holding time (min), flow rate of the mixture solvent (mL/min) and ethanol concentration (%). The RSM generated 30 different experiments with 6 replicates at the center point (Table 3.2). For all the extractions, pressure inside the reactor was maintained at 15MPa. During the sCF extraction, only the experimental temperature varied slightly from the theoretical value obtained from the software. The other variables were the same and were maintained constant during the experiments.

Table 3.2. Response surface methodology for the sCF (aqueous ethanol) extraction

Run	Theoretical				Experimental
	Temperature (°C)	**Static holding time (min)**	**Flow rate (mL/min)**	**Ethanol (%)**	**Temperature (°C)**
1	150	2	4	10	149-151
2	135	16	3	15	133-137
3	165	7	3	15	163-167
4	165	16	3	15	163-167
5	150	11	4	20	149-152
6	135	7	3	15	131-139
7	165	7	3	5	163-170
8	165	16	5	15	163-170
9	150	11	4	0	149-151
10	135	7	3	5	131-139
11	135	16	5	15	131-139
12	165	7	5	15	163-170
13	165	16	3	5	163-170
14	135	7	5	15	131-139
15	165	16	5	5	163-170
16	150	20	4	10	149-151
17*	150	11	4	10	149-152
18	135	7	5	5	131-139
19*	150	11	4	10	149-152
20	165	7	5	5	163-170
21	120	11	4	10	118-125
22	150	11	6	10	149-152
23	180	11	4	10	175-185
24	135	16	3	5	131-139
25*	150	11	4	10	149-152
26	150	11	2	10	149-152
27	135	16	5	5	131-139
28*	150	11	4	10	149-152
29*	150	11	4	10	149-152
30*	150	11	4	10	149-152

* Center point (Temperature = 150°C; Static holding time = 11 min; flow rate = 4mL/min; and ethanol concentration = 10%).

3.3.3.1.2. Extraction using ILs

(a) Synthesis of the IL (Preparation)

A simple acid-base neutralization reaction takes place during the formation of the Brönsted IL (Bicak, 2005) as shown in Fig. 3.2. The m-2-HEAA was prepared using the method discussed by Alvarez et al. (2011) with slight modifications.

For the preparation, a triple necked glass flask equipped with a reflux condenser and a dropping funnel was mounted inside an ice bath with NaCl. Then, 2-(Methylamine) ethanol was loaded and acetic acid was then added drop by drop to the flask using a funnel under continuous stirring with a magnetic stirrer. Since this chemical reaction is highly exothermic, care was taken to adequately use low temperature (0°C) throughout the synthesis to avoid the dehydration of the salt. At the end of the reaction, additional mixing with the stirred was performed for 4 h at room temperature, and then the mixture was heated to 70°C for 17 h with continuous stirring until the final viscous liquid with a yellow color was obtained. Since the prepared m-2-HEAA is hygroscopic, before storage approximately 500mL of the IL was dried for 8 h at 70°C using a vaccum pump and nitrogen was flushed into the mixture after every 2h. Before each use, the same drying process was performed for 2h. The humidity of the IL (0.32%) was determined using the Karl Fischer titration method in the Chemistry Department at the University of Alberta.

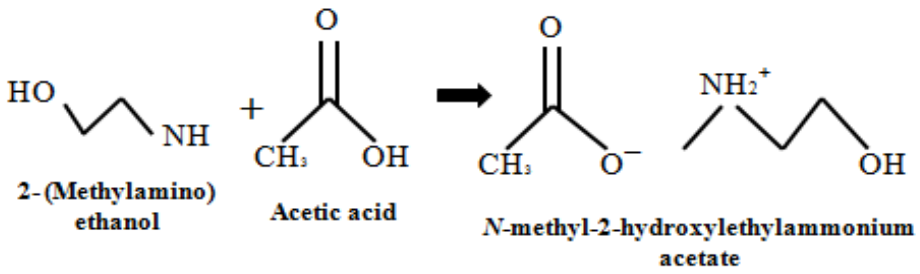


Figure 3.2. Acid-base neutralization of m-2-HEAA Brönsted ionic liquid synthesis (adapted from Alvarez et al., 2010).

(b) Solid-IL batch extraction

Pure m-2-HEAA was used for the batch extraction,varying temperature (70-120°C), IL:barley hull ratio (10-50%) and time (2-6 h). The experiments were performed following the methodology described by Fu et al. (2010) where 0.5g of barley hull was incubated with the appropriate amounts of 2-HEAA at the specified temperature using a heater. The mixture loaded inside a 30mL beaker was constantly mixed using a magnetic stirrer (approximately 25-30 rpm) under a flow of nitrogen. The temperature was monitored using a thermometer. After carrying the reaction at specific conditions, the suspension was diluted by 100mL of 0.1mol/L NaOH. The solution was then centrifuged for 20 min and the supernatant was decanted and stored in glass vials. The supernatant was cooled before analyzing the responses for total carbohydrates and phenolics.

The optimization process used RSM with a central composite design that generated 20 experiments (see Table 3.3). The different variables investigated were temperature, IL:barley hull ratio and time on the extraction of total carbohydrates and phenolics.

Table 3.3: Response surface methodology for the solid-IL batch extraction

Run	Theoretical			Experimental
	Temperature (°C)	IL:Barley hull (%)	Time (h)	Temperature (°C)
1*	95	30	4	90-100
2	95	10	4	90-100
3	120	30	4	115-125
4	95	50	4	90-100
5	110	42	3	105-115
6	110	18	5	105-115
7	95	30	2	90-100
8*	95	30	4	90-100
9	80	42	5	75-85
10	95	30	6	90-100
11*	95	30	4	90-100
12	80	18	5	75-85
13	110	18	3	105-115
14	70	30	4	65-75
15	80	42	3	75-85
16	80	18	3	75-85
17*	95	30	4	90-100
18*	95	30	4	90-100
19	110	42	5	105-115
20*	95	30	4	90-100

*center point (Temperature = 95°C; IL:barley hull ratio = 30%; and time = 4 h)

(c) Extraction using aqueous IL at subcritical conditions

The IL selected during this study was from the hydroxyl ethylammonium due to its low cost and simple synthesis procedure by the modification of the aliphatic chain of the organic acid. In addition to these advantages, the negligible toxicity of the m-2-HEAA was also an important factor (Yuan et al., 2007).

Further studies were done to compare the efficiency of using aqueous solution of m-2-HEAA under subcritical conditions for the extraction of total carbohydrates and phenolics. The experiments were performed using the same sCF procedure discussed in Section 3.2.2.2. A simple orthogonal array was

used to study the effect of temperature (100 and 160°C), static holding time (2 and 15 min) and aqueous IL solution (20 and 50%).

3.3.2.3.Supercritical carbon dioxide (SC-CO_2) extraction

A laboratory-scale *SC-CO_2* system equipped with a 150 mL extraction cell (Supercritical Technologies Inc., Newark, NE, USA) was used for the extractions (Figure 3.3). The extraction temperature was monitored by a thermocouple immersed in the band heater. The procedure for the extraction was the same as described earlier by Prado et al. (2011). Barley hull (5g) was first loaded in the reactor. The CO_2 was pumped to the system until extraction pressure was reached and the pressure was maintained constant. The CO_2 flow rate (1mL/min, measured at ambient conditions) was controlled by a micrometering valve. During the extraction, ethanol was used as a co-solvent with the flow rate of 2 mL/min. Ethanol (50% v/v) was introduced continuously using a HPLC pump and the flow rate was maintained constant. Each extraction was carried out for 3h. Extract fractions were collected every 30 min in glass vials held in an ice-pack kept at around -20°C. The ethanol in the extracted material was then evaporated under gentle nitrogen flow. The samples were weighed and stored at -18°C until further analysis. At least two replicates were carried out for each experiment. The experiments were conducted at temperatures of 60 and 120°C and a pressure of 30MPa.

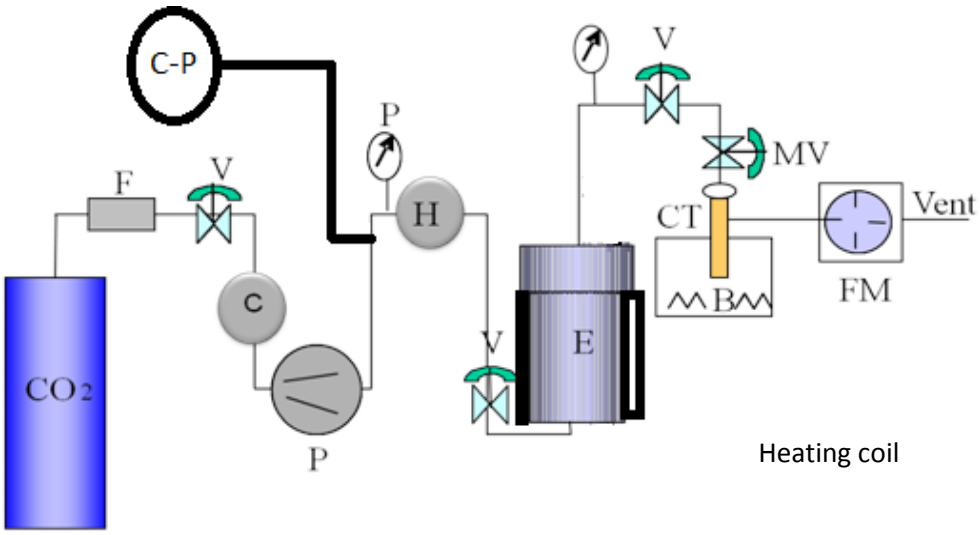


Figure 3.3: SC-CO_2 extraction system. (F: filter, V: on/off valve, P: pump, C-P: co-solvent pump, P: pressure gauge, E: extraction vessel with heater, T: thermocouple, MV: micrometering valve, CT: collection tube, B: cold bath, FM: gas flow meter, C: cooler, H: Heater)

3.3.3. Analysis of the extracts

3.3.3.1.Characterization of the extracts

The extract solutions obtained from the solid-liquid and subcritical fluid extraction methods were characterized on the basis of their pH, refractive index (RI) and conductivity. No characterization was performed for the SC-CO_2 extracts.

3.3.3.1.1. pH and conductivity measurements

The pH of the extracts was measured at room temperature (22°C) using an Excel XL50 pH/Conductivity Meter (Fisher Scientific Accumet, Brightwaters, NY, USA). The electrodes for the pH and conductivity measurements were a liquid-filled probe and a four-cell conductivity nominal constant k=1 (Fisher Scientific Accumet, Brightwaters, NY, USA), respectively. The uncertainties for the pH and conductivity measurements were ±0.05 and ±10 μS/cm, respectively.

3.3.3.1.2. Refractive index (RI)

The RI of the extracts was measured at 20°C using an automatic refractometer Mettler-Toledo RE50 (Tokyo, Tokyo, Japan) with a resolution of $\pm 10^{-5}$, an uncertainty in the experimental measurements of $\pm 2x10^{-5}$ and an uncertainty in the temperatures of ±0.01°C. The unit has a Peltier technology to keep the temperature constant.

3.3.3.1.3. Color measurements

The color of the extract solutions were analyzed using a Minolta CR-400 Colorimeter (Konica Minolta Sensing Americas, Inc., Ramsey, NJ, USA) known as the Hunter Lab colorimeter. The colorimeter was primarily calibrated on a solid white tile. The cuvettes were filled up to half of its volume with extract solutions and placed on the solid white color tile. The readings were taken using an illuminant D65 as the source of light and expressed in terms of *L* (lightness), *a* (redness) and *b* (yellowness) values. The *L* runs on the z-axis and an *L* value of 100 is perfect white while an *L* value of 0 is perfect black. The *a* component is in the x-axis with positive values being more red and negative values being more green, and the *b* component is in the y-axis with positive values being more yellow and negative values being more blue (Papadakis et al., 2000).

3.3.3.1.4. Measurement of brown color formation

The extracts were placed in plastic cuvettes of 1.5mL and the absorbance was read at 294 and 420nm using a spectrophotometer (model DU-800, Beckman Coulter Ltd., London, UK). The measurement of the brown color formation was used as an efficient and simple method for monitoring the non-enzymatic browning reactions, with consideration to the Maillard

reactions (Plaza et al., 2010). Measurement of brown color formation in barley hull extracts was achieved at 420 nm (Kato et al., 1986; Labuza and Mamaro, 1990). Intermediate stages in the non-enzymatic browning reactions were measured at the UV-absorbance of 294 nm (Ajandouz et al., 2001). Results were calculated using the formula reported by Smith et al. (1990).

$$\text{Absorbance (A, mL/g)} = \frac{B \times V}{W} \quad (3.7)$$

where, B = absorbance of the extract,

V = volume of the extract (~100mL), and

W= weight of the initial sample (g)

3.3.3.1.5. Total carbohydrate analysis

Total carbohydrate content was determined using the methodology of Dubois et al. (1956) for all the extracts obtained in this thesis. Dilutions of the extracts were performed depending on the concentration of each extract sample. Then, 0.5mL of phenol and 2.5mL of sulfuric acid (96%) was added to an aliquot of 1mL of the diluted extract. Good mixing was attained by vortexing each sample for approximately 1 min. The reaction was then stopped using a cold water bath at 20^{o}C for 20 min. The calibration curve for total carbohydrates was prepared using glucose solutions ranging from 0.5 to 10 mg/g of solution. The absorbance was measured at 490 nm using a spectrophotometer (Genova MK3, New Malden, Surrey, UK). The final results were expressed as milligrams of glucose equivalents per gram of barley hull.

3.3.3.1.6. Total phenolics analysis

Total phenolics of barley hull extracts were determined following the methodology of Singleton and Rossi (1965), with minor modifications.

Briefly, 0.04mL of extract was mixed with 3.16mL of distilled water and 0.1mL of Folin-Ciocalteau's phenol reagent and the mixture was vortexed thoroughly. After 6 min of reaction, 0.3 mL of sodium carbonate solution was added to the mixture. The mixture was then incubated at room temperature for 2 h. After incubation in a dark place, the absorbance was read at 765nm and the measurements were compared with the calibration curve of gallic acid solutions. Total phenolics were expressed as milligrams of gallic acid equivalents per gram of barley hull.

3.3.3.1.7. Total antioxidant activity

The total antioxidant activity of the extract was measured by 2,2-DiPhenyl-1-PicrylHydrazyl (DPPH) and Ferric Reducing/Antioxidant Power (FRAP) assay.

(a) DPPH assay

The methodology of Blois (1958) previously described by Gülçin (2006) was used with slight modifications to assess the DPPH free radical scavenging capacity of the extracts. Briefly, 19.71 g of DPPH was dissolved in 500mL of ethanol to prepare 0.1mM solution of DPPH. Then, 1.5mL of this DPPH solution was added to 0.5mL of barley hull extract. These solutions were vortexed thoroughly and incubated in a dark environment at room temperature for 1 h. The blank samples (Milli-Q water or aqueous ethanolic solutions) were also prepared using the same method. Then, the absorbance of these mixtures was measured at 517 nm using a Genova spectrophotometer (Genova MK3, New Malden, Surrey, UK). The decrease in absorbance of a sample was calculated in comparison with the DPPH solution. Measurements were performed in duplicate. The antioxidant activity, defined as the

percentage of the DPPH free radical, was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{A - B}{A} \times 100 \qquad (3.8)$$

where, A = absorbance of the DPPH control solution (0.1mM DPPH solution), and

B = absorbance of the DPPH control solution + absorbance of the extract.

(b) FRAP assay

FRAP analysis was performed according to the methodology reported by Benzie and Strain (1996) with some minor modifications. This method considers the ferric to ferrous ion reduction at low pH, which results in a blue colored ferrous-tripyridyltriazine complex. FRAP values are obtained by comparing the absorbance change at 593nm in a test reaction mixture with those containing ferrous ions at known concentrations. At low pH, the FRAP assay depends on the extent of the reduction of ferric tripyridyltriazine (Fe (III)-TPTZ) to the ferrous tripyridyltriazine (Fe (II)-TPTZ) in the presence of a reductant (antioxidant or other reducing agent). The Fe (II)-TPTZ complex has an intense blue color which can be read using a spectrophotometer at a wavelength of 593nm (Benzie and Strain, 1996).

The FRAP solution was prepared by mixing 47.5mL of buffer acetate of pH 3.6, 4.75mL of 10mM TPTZ solution and 4.75mL of 20mM ferric chloride solution. The FRAP solution (3mL) reacted with 0.1mL of the extract solution and 0.3mL of water. The solution was then incubated at 37°C in a water bath for 30 min. Absorbance of the colored product was then read at 593nm. The calibration curve was prepared using ferrous sulphate solutions

and the results were expressed in moles of ferrous sulphate per gram of sample. Additional dilutions were used when the reading of the FRAP solution was higher than the linear range of the calibration curve.

3.3.4. Kinetic modeling

The kinetic curve for the sCF extraction process was fitted with the model reported by Kandiah and Spiro (1990). This model assumes that the extraction rate is limited either by internal diffusion or by external elusion of the solute. The extraction has two simultaneous extraction rates according to the following equation:

$$\frac{C_i}{C_o} = 1 - \left[f_1 \exp(-k_1 t) - (1 - f_1) \exp(-k_2 t) \right] \qquad (3.9)$$

where, C_i = concentration at the time i,

C_o = total concentration of the solute,

f_1 = fraction of solute extracted at rate constants k_1 and k_2, respectively.

t = time

Parameters of the kinetic model (f_1, k_1 and k_2) were calculated by regression analysis of the experimental data. The minimization of the objective function used a genetic algorithm code. The deviation of the model fitting was calculated using the mean square error (*mse*):

$$mse = \frac{100}{N} \sum_{i=1}^{N} \left[\frac{(x^{cal} - x^{\exp})_i^2}{N - m} \right] \qquad (3.10)$$

where, x = property to fit ($x_i = C_i / C_0$),

m = number of parameters of the model,

N = number of data points,

exp = experimental data

cal = calculated data

3.3.5. Statistical analysis

The software Design Expert version 6 was used for the statistical analysis of the different parameters for the solid-liquid and subcritical fluid extraction methods. All the experiments were performed in a randomized order. The design consisted of 6 replicates at the center point. The validation of the model was done using the analysis of variance (ANOVA).

Chapter 4: Results and Discussions

The results presented in this study maximized extraction of total carbohydrates and phenolics from barley hull using different extraction methods. In addition, extracts were characterized on the basis of pH, RI, conductivity, color and their antioxidant activities to assess the effect of different process conditions, such as temperature, ethanol concentration, pH, flow rate and static holding time.

4.1. Proximate compositional Analysis

The proximate compositional analysis of BT 584 variety of barley hull is reported for the first time (Table 4.1). The moisture, fat, ash and protein contents obtained in this research had little variation with the data reported in the literature as shown in Table 4.1.

The moisture content of BT 584 hull was low (7.54% on wet basis). Krawczyk et al. (2008) reported that the total solid content of barley hulls (variety not specified) was 92%, therefore the amount of moisture in barley hull accounted for 8% of the total weight. The ash content of the barley hull in this study was 8.18%, which was similar to the result obtained by Krawczyk et al. (2008). However, an average ash content of 15.49% was reported by Garrote et al. (2008), with increasing ash content for samples with smaller particle size. The fat content of BT 584 hulls during this study was 1.45%, while a value of 3% fat content was reported for barley hulls from a variety named Cindy or waxy barley (Hoije et al., 2005). The protein content analyzed in this study was 5.53%; however, 4.45-10.65% of protein content was reported in barley hull (Garrote et al., 2008; Hoije et al., 2005; Parajo et al.,

2004), which is in agreement with this study. The total phenolics obtained in this study (13.102±0.169 mg/g) were not similar to the 33-36mg/g in barley hull obtained by Garrote et al. (2008), possibly due to the high temperature of autohydrolysis (190-229°C) used by Garrote et al. (2008). In this study, the free phenolics was in the range of 9.833±0.099 mg/g while the bound phenolics were 3.268±0.239 mg/g. The total carbohydrate was the most important component of the barley hull (~75%). Barley hull in this study was composed of a high percentage of cellulose (32.6%), followed by hemicellulose (26.08%) and lignin (14.26%).

The differences in the values obtained in this study with the values reported in the literature are probably due to the different barley varieties used in the studies and different methodologies used for those analyses. For example, Garrote et al (2008) used autohydrolysis for the extraction of phenolics from barley hull. Thus, their recovery was higher as compared to the phenolics obtained from alkaline hydrolysis during this study. Thus, different organic solvents and methods influence in different ways to the reliability of the proximate compositional analysis.

Table 4.1 Proximate compositional analysis of BT 584 hull

Component	This study	Literature	Reference
Moisture (%)	7.537±0.006	8-10	Olkku et al. (2005)[a], Krawczyk et al. (2008)[a]
Ash (%)	8.187±0.001	9.1	Olkku et al. (2005), Krawczyk et al. (2008)
Fat (%)	1.445±0.012	1-2	Hoije et al. (2005)[b], Olkku et al. (2005)[a]
Protein (%)	5.533±0.006	7-10	Garrote et al. (2008)[a], Hoije et al. (2005)[b], Parajo et al. (2004)[a]
Total phenolics (mg/g hull)	13.102±0.169	33-36	Garrote et al. (2008)
Total carbohydrates (%)	75.148±0.034	85-90	Olkku et al. (2005)
Cellulose (%)	32.66±0.116	26.5-28.8	Olkku et al. (2005), Moldes et al. (2002)[a], Hoije et al. (2005)
Hemicellulose (%)	26.08±0.004	33.5-33.7	Olkku et al. (2005)
Lignin (%)	14.26±0.248	21.4-22.8	Olkku et al. (2005), Moldes et al. (2002), Hoije et al. (2005)

'a' means that the barely hull variety was not specified; 'b' stands for barley hull produced from cindy or waxy barley variety

4.2. Solid-liquid extraction

The solid-liquid extraction has long been used to extract phenolic compounds from different plant sources (Liu and Yao, 2007). Thus, in this study, the effectiveness of using aqueous ethanol at different concentrations (0-80%) as a solvent was investigated for the extraction of carbohydrates and phenolics. Roman et al. (2009) showed that 80% ethanol was effective to extract more β-glucan from barley flour (~87.7% of β-glucan). In addition, Liu et al. (2006) reported that 70% ethanol was enough for the extraction of total phenolics from barley grain. Moreover, temperature and pH had positive effects on the extraction of carbohydrates and phenolics from flax shives as reported by Liu and Yao (2007). The high temperature (70°C) and high pH (12) of water showed to affect the fractionation of non-polar components (lignin) and a maximum amount of lignin was liberated from flax shives at

high temperatures by breaking the esterified bonds of lignin (Kim and Dale., 2004). A temperature of 55°C of the aqueous ethanol solvent showed to be efficient for the extraction of β-glucan from barley (Roman et al., 2009).

Previous studies in our lab with lentil husk have also evaluated the efficiency of using the aqueous ethanol solutions for the extraction of bio-molecules, such as carbohydrates and phenolics (Saldaña et al., 2012). Thus, the variables studied in this thesis for the optimization of the RSM of the solid-liquid extraction of barley hull were temperature (50-70°C), ethanol concentration (0-80%), and pH (4-12). The RSM generated 20 different experiments with different possible experimental conditions (as discussed earlier in Table 3.1), which was then used to determine the best condition for the extraction.

The time of extraction was determined as a function of the refractive index (RI) values at the center point (60°C using 41.2% aqueous ethanol solution with a pH of 8.0). The extracts were collected every 10 min to measure the RI value (Fig 4.1). After 180 min of extraction, there was no significant change in the RI value. Thus, all the extractions were performed for 180 min.

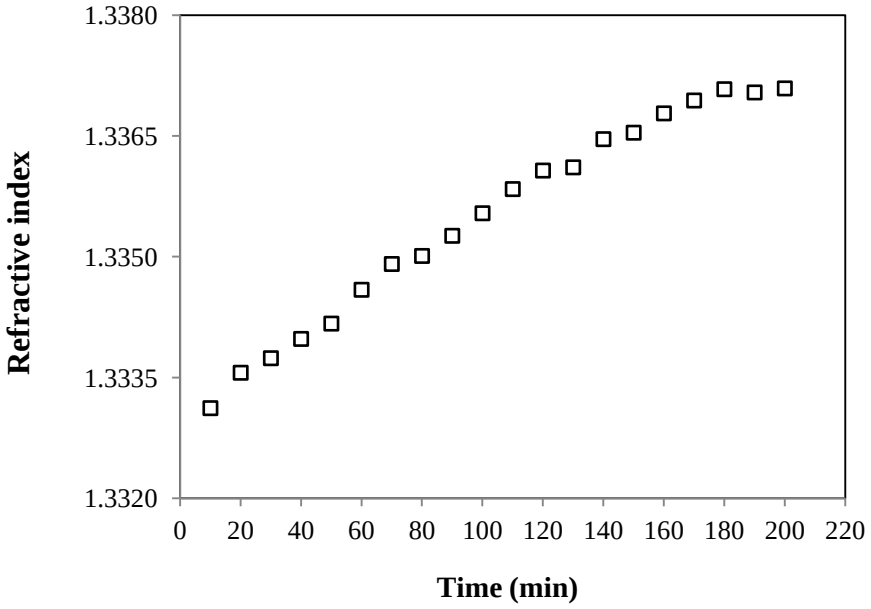


Figure 4.1. RI as a function of time for the solid-liquid extraction performed at 60°C with 41% ethanol and pH of 8.

4.2.1. *Characterization of the extracts*

The physico-chemical properties of the extracts, such as *pH*, *RI*, conductivity (*k*), and color measurement as well as the total carbohydrates content, total phenolics content and antioxidant activity using DPPH and FRAP analysis are reported in Table 4.2.

It was observed that the pH of the extract solution after 3h extraction increased slightly compared to the initial pH of the solution. For example, the experiment carried out at 70°C with an initial pH of 8 resulted in a solution with a pH of 8.8 after 3 h of extraction. Similar trends were observed for the other experiments performed for the solid-liquid batch extractions (Table 4.2). This little change in the pH showed that the use of a low temperature during the extraction avoided the formation of any acidic compound. In addition, Table 4.2 showed that the increase in temperature from 50 to 70°C extracted

more total carbohydrates (9.8 to 13.9mg/g of barley hull, respectively) and resulted in increased RI and κ values as high amounts of total solids were removed. The RI of the extracts changed from 1.3333 to 1.3365 due to the small change in experimental temperature from 50 to 70°C using 41% ethanol concentration and pH of 8, respectively. The *k* value also changed with the change of the temperature from 50 to 70°C (125.8 to 694.8µs/cm, respectively).

The color of the extract solutions can be attributed to the less formation of brown colored compounds due to the low temperatures used for the extractions. The development of the brown color was effectively used as a method to monitor the occurrence of any non-enzymatic browning reactions, such as Maillard reactions (Delgado-Andrade et al., 2010; Purlis et al., 2010). The high value of *L* (more than 90) for all the extract solutions showed that the extract was lighter in color. The *b* value of the extract solution had a high positive value ranging from 11.01 to 15.41, indicating that the extract had a light yellow color which was also apparent from the visual observation of the extract solutions (Fig 4.2).

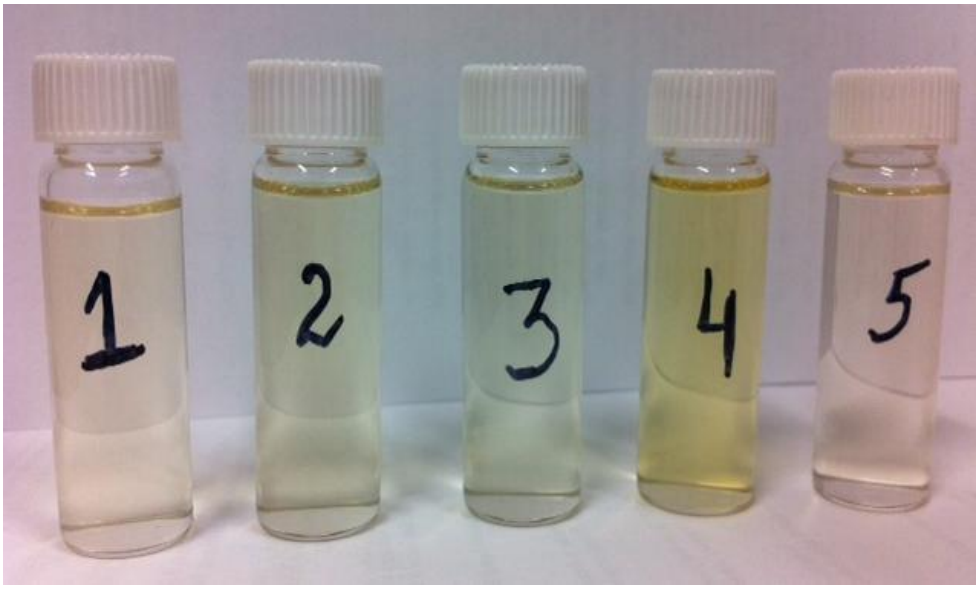


Figure 4.2. Extracts collected with 41% ethanol concentration and pH of 8 at 50°C (1), 60°C (2) and 70°C (3); with 41% ethanol, 60°C and pH of 12 (4); and with 83% ethanol, 60°C and pH of 8 (5).

The increase on the browning color can be associated with advanced phases of the Maillard reactions (Morales and Jime, 2001). Thus, the absorbances at 290 and 420 nm were commonly employed to monitor the formation of the brown advanced Maillard reaction products. The highest absorbance value at 420nm (28.3 mL/g) was obtained at 70°C, ethanol concentration of 41% and pH of 8 (experiment 15 in Table 4.2). The temperature had a positive effect on the Maillard reaction. The 20°C rise in temperature from 50 to 70°C showed to be more effective for the formation of browning products (as shown in Table 4.2).

On the other hand, higher ethanol concentration (83%) resulted in approximately 11.9 mg of total phenolics/g of barley hull, which is higher than 6.9 mg/g at 0% ethanol concentration at 60°C and pH of 8. Thus, higher ethanol concentration had a positive effect on the phenolics extraction. At the highest temperature of 70°C, with 41.2% ethanol concentration and pH 8.0,

the total carbohydrates and phenolics were 13.9 and 10.6 mg/g of barley hull, respectively. The experiment conducted with the highest amount of ethanol concentration (83%) showed the maximum DPPH and FRAP values of 30.4% and 75.7mMol Fe_2SO_4/g of barley hull, respectively. Thus, it was observed that the increase in the phenolics content was directly proportional to the antioxidant activity of the extract solution.

The change in the pH during the experiment had no significant effect on the extraction of total phenolics and total carbohydrates (Refer to Table 4.3).

Table 4.2 Response values for the solid-liquid batch extraction**.**

Run	Solvent			Extract solution											
	Theoretical/Experimental			Physical properties								Total		Antioxidant Activity	
	*T*** (°C)	Ethanol (%)	*pH*	*pH*	*RI***	*κ*** (µs/cm)	294nm (mL/g)	420nm (mL/g)	*L*	*a*	*b*	CHO** (mg/g)	Phe** (mg/g)	DPPH** (%)	FRAP (mMol FS**/g)
1	60.0/60.0	0/0	8.0/8.0	8.03	1.33369	113.6	78.3	12.3	96.41	-1.41	12.61	9.37	6.94	23.7	44.3
2*	60.0/60.0	41.0/41.3	8.0/8.0	7.93	1.33344	470.8	96.4	12.4	95.41	-1.11	12.44	10.52	9.45	27.3	48.9
3	60.0/60.0	41.0/41.3	12.0/12.0	12.02	1.33333	574.9	189.7	13.4	95.96	-2.41	13.01	11.46	10.28	1.0	68.7
4	54.0/54.0	17.0/16.7	6.0/5.62	6.41	1.33327	122.7	71.0	7.7	94.11	-2.46	13.46	9.74	8.22	23.9	43.5
5	66.0/65.9	17.0/16.7	6.0/5.62	5.96	1.33348	611.4	80.7	9.1	95.16	-2.11	13.74	10.79	9.55	24.2	46.6
6	60.0/60.0	83.0/82.50	8.0/8.0	8.01	1.33321	446.8	123.4	3.1	96.07	-2.34	14.71	12.31	11.93	30.4	75.7
7	50.0/50.0	41.0/41.2	8.0/8.0	8.46	1.33334	125.8	81.1	11.4	98.11	-2.61	15.41	9.18	7.34	24.5	44.4
8*	60.0/60.0	41.0/41.3	8.0/8.0	8.12	1.33378	187.1	103.4	15.6	99.11	-2.77	13.41	11.81	9.14	27.0	50.1
9	54.0/54.0	17.0/16.7	10.0/10.4	10.58	1.33346	116.7	73.0	12.4	98.41	-1.98	12.11	8.35	8.39	19.8	50.9
10	60.0/60.0	41.0/41.3	4.0/4.0	4.66	1.33387	125.4	85.3	4.2	95.44	-1.99	12.98	10.78	8.82	24.9	45.7
11	54.0/54.0	66.0/65.8	6.0/5.6	6.02	1.33485	137.4	107.5	4.2	95.12	-3.41	12.94	9.26	7.84	26.3	44
12*	60.0/60.0	41.0/41.3	8.0/8.0	6.11	1.33659	133.6	103.8	19.1	96.12	-3.64	12.34	8.74	9.36	25.3	49.1
13	66.0/65.9	66.0/65.8	10.0/10.4	10.78	1.33334	514.3	95.5	17.2	95.99	-2.22	13.71	12.01	9.48	23.2	48.9
14*	60.0/60.0	41.0/41.3	8.0/8.0	7.98	1.33302	126.4	95.1	15.2	92.41	-2.12	13.84	10.16	8.28	26.6	45.6
15	70.0/70.0	41.0/41.3	8.0/8.0	8.77	1.33658	694.8	109.5	28.3	94.46	-2.98	11.46	13.94	10.59	25.5	47.1
16*	60.0/60.0	41.0/41.3	8.0/8.0	7.12	1.33485	167.4	90.7	14.2	93.21	-2.2	11.01	9.66	9.23	29.7	46.9
17	66.0/65.9	17.0/16.7	10.0/10.4	10.45	1.33332	499.4	89.4	16.0	94.11	-2.16	12.04	11.95	9.36	24.1	43.2
18	54.0/54.0	66.0/65.8	10.0/10.4	10.01	1.33389	127.8	109.7	11.4	95.06	-2.87	12.11	9.47	8.96	24.3	54.6
19*	60.0/60.0	41.0/41.3	8.0/8.0	7.59	1.33785	199.4	88.2	17.1	95.41	-2.89	12.46	9.91	8.93	26.6	43.1
20	66.0/65.9	66.0/65.8	6.0/5.6	6.48	1.33345	145.6	199.6	7.0	95.22	-2.3	12.41	11.99	8.94	26.4	54.4

T: temperature, RI: refractive index, κ: conductivity, CHO: total carbohydrates, Phe: total phenolics, DPPH: 2,2-DiPhenyl-1-PicrylHydrazyl, FRAP: Ferric Reducing/Antioxidant Power, FS: Fe_2SO_4,**

*Center point: mean ± uncertainty: pH =7.47±0.76; RI =1.3355±0.001; κ =214.17±128.9; color absorbance at 294nm =96.27±6.40; color absorbance at 420nm =15.6±2.31; Hunter LAB: *L*= 95.28±2.36, *a* = -2.455±0.88, *b* = 12.58±0.98; Carbohydrates (mg/g) = 10.35±0.88; Phenolics (mg/g) = 9.1±0.68, DPPH = 27.08±1.45, FRAP = 47.28±2.62.

4.2.2. *ANOVA analysis*

The coefficients of the statistical model and the ANOVA analysis for the experiments used for the influence of the solid-liquid batch extraction of each factor such as temperature (A), concentration of ethanol (B) and pH (C) was obtained using the response surface methodology (Table 4.3).

Table 4.3 showed that temperature, and ethanol concentration had no significant effect on the pH of the extract solution. The same observation was obtained for the refractive index, which was approximately equal to 1.333 for all the extracts and the change of variables had no effect on this parameter. The increase of the conductivity from 125.8 to 694.8µs/cm was influenced by the increase of the temperature (50 to 70°C) and pH of the solvent (8 to 12). The pH (4 to 12) had a significant influence on the change of color measured at 420nm which changed from 4.1 to 13.4mL/g.

It was also observed that the increase of all the three variables (temperature, ethanol concentration and pH) had a positive effect on the extraction of carbohydrates (8.35 to 13.94mg/g), phenolics (6.94 to 11.93 mg/g) and the antioxidant activity calculated using the FRAP method (43.1 to 75.7mMol Fe_2SO_4/g of barley hull). It has been already reported that the high ethanol concentration was essential for the recovery of phenolics from barley hull, mainly bound phenolics which are released from the complex matrix (Yu et al., 2002). The same effect was observed during this study.

Table 4.3: ANOVA for the statistical model for the responses of the solid-liquid batch extraction

Responses	**pH****		**RI****		**K****		**420nm**		**CHO****		**Phe****		**FRAP****	
Model	***quadratic***	***p***	***quadratic***	***p***	***quadratic***	***p***	***quadratic***	***p***	***quadratic***	***p***	***linear***	***p***	***linear***	***p***
Intercept	7.48		1.3355				15.64		10.17		9.05		49.79	
A	0.09	0.57	-0.0002	0.62	*153.77*	*<0.01*	1.24	0.16	*1.30*	*<0.01*	*0.68*	*0.01*	0.34	0.86
B	-0.01	0.94	0.0001	0.81	23.12	0.57	-1.53	0.09	0.50	0.09	*0.59*	*0.02*	*5.16*	*0.02*
C	*2.15*	*<0.01*	-0.0001	0.69	*86.23*	*0.05*	*5.09*	*<0.01*	0.08	0.75	0.31	0.19	3.50	0.09
A^2	0.39	*0.02*	-0.0008	*0.05*			-1.40	0.11	0.33	0.23				
B^2	0.18	0.22	-0.0007	0.08			-3.06	*0.00*	0.08	0.76				
C^2	0.30	0.06	-0.0006	0.11			-0.04	0.96	0.18	0.49				
AB	0.23	0.26	-0.0003	0.59	-37.02	0.48	0.45	0.68	0.08	0.82				
AC	0.08	0.69	0.0001	0.89	56.60	0.29	0.65	0.56	0.27	0.45				
BC	-0.05	0.81	-0.0001	0.77	82.20	0.13	0.73	0.51	0.06	0.86				

**RI: refractive index, κ: conductivity, CHO: total carbohydrates, Phe: total phenolics, FRAP: Ferric Reducing/Antioxidant Power, FS: Fe_2SO_4

$p \leq 0.05$ means the factor is significant; $p \geq 0.05$ means the factor is non-significant.

A = Temperature (°C), B = Ethanol concentration (%) C = pH

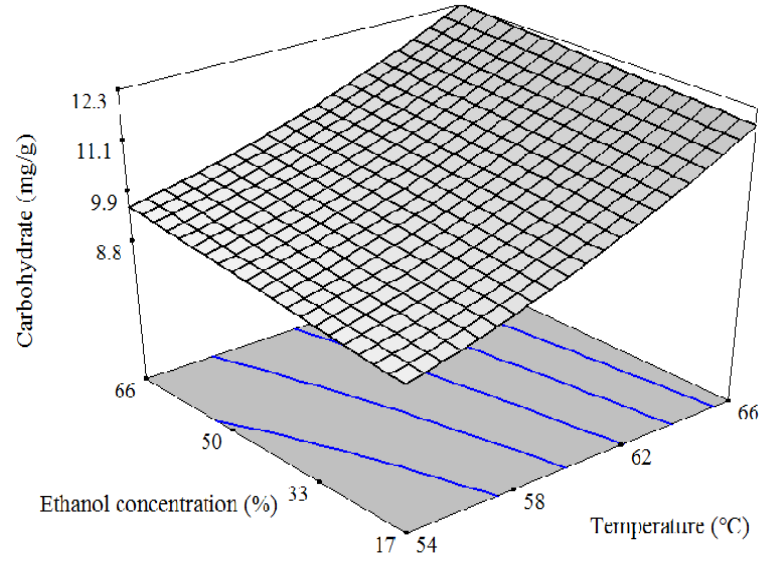
Since this study was based on the maximization of total carbohydrates and phenolics extraction, the statistical analyses for the total phenolics and carbohydrates for each set of variable combinations for solid-liquid batch extraction were obtained. The analysis showed that the response of total carbohydrates and total phenolics fitted well to the second order polynomial model and linear model, respectively (equations 4.1 and 4.2).

$$\text{Total Carbohydrates (mg/g)} = 10.35 + 1.47A + 0.31B + 0.044C + 0.30A^2 - 0.008B^2 + 0.18C^2 - 0.11AB - 0.057AC + 0.16BC \quad (p = 0.0071) \qquad (4.1)$$

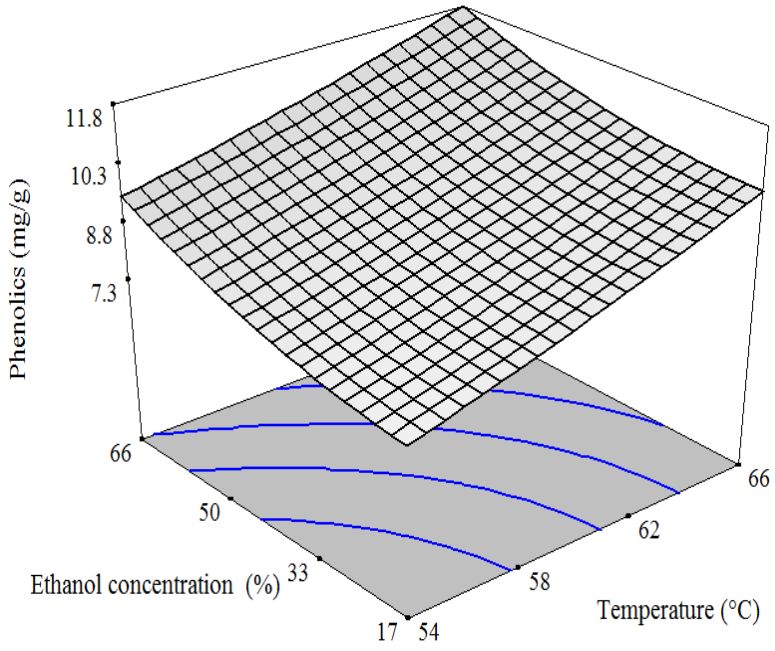
$$\text{Total Phenolics (mg/g)} = 9.65 + 1.30A + 0.95B + 0.25C \quad (p = 0.0077) \qquad (4.2)$$

where, A, B and C are the variables indicated in Table 4.2.

The model had satisfactory accuracy based on p values (R^2 equal to 0.830 and 0.515 for total carbohydrates and phenolics, respectively). Temperature (p= 0.01) and ethanol concentration (p= 0.02) were found to be the significant variables (Fig. 4.3 and Table 4.3)



(a)



(b)

Figure 4.3 (a) Total carbohydrates (mg/g) and, (b) total phenolics (mg/g) extraction as a function of temperature and ethanol concentration for solid-liquid batch extraction of barley hull

Solid-liquid batch extraction was performed at the optimized conditions of 70°C, ethanol content of 82.5% and pH of 12. The yields obtained at these optimized conditions were 14.01±0.125 mg of total carbohydrates/g of barley hull and 13.44±0.016 mg of total phenolics/g of barley hull based on duplicate runs. The values of the experimental recoveries were in agreement with the predicted recovery values (14.61mg of total carbohydrates/g of barley hull; 13.86mg of total phenolics/g of barley hull) using the response surface methodology. These results were further used for the comparison of the different extraction methods.

4.3. Subcritical fluid (sCF) extraction (water+ethanol)

The sCF extraction was done in two different stages and then further analysis was done on the basis on these two stages. The first stage of experiments was to carry out preliminary experiments to study the influence of some of the most important parameters (temperature, pressure, static holding time, particle size). After obtaining the significant parameters, a second set of experiments were conducted to obtain the optimal values of the significant parameters using the RSM.

4.3.1. Preliminary study

For the preliminary study, temperature, pressure, particle size and static holding time were considered as the main factors that influenced the extraction. For all the experiments, water with a flow rate of 2mL/min was used to ensure maximum internal and external diffusion through the matrix inside the reactor (Singh and Saldaña, 2011). All these factors were analyzed on the basis of total

carbohydrates and phenolics extraction. The effects of different parameters are discussed in detail in the following sections.

4.3.1.1. Effect of temperature

Total carbohydrates and phenolics were extracted at different temperatures as shown in Fig 4.4 (Appendix C.1). It was observed that the extraction of total carbohydrates and phenolics increased with the increase in temperature. The importance of the use of high temperature was observed during the solid-liquid batch extraction (Fig 4.3). The change in temperature from 25 to 250°C changed properties of water during the extraction, such as decrease of the permittivity, viscosity and surface tension while increases its diffusivity (Teo et al., 2010). The increase of temperature from 100 to 250°C decreases the viscosity (from 281.74 µPas to 106.11 µPas), thermal conductivity (0.679 W/mk to 0.621 W/mk) and surface tension (0.059 N/m to 0.026 N/m) (NIST, Chemistry Webbook).

At 120°C, 15MPa and 180 min, the amounts of total carbohydrates and phenolics were about 33.6 and 7.0 mg/g of hull, respectively. Increasing the temperature to 180°C, these amounts of total carbohydrates and phenolics increased significantly to about 113.6 and 81.3 mg/g of barley hull, respectively. The extraction efficiency at high temperatures using subcritical water extraction may be attributed to the higher solubility of carbohydrates in subcritical water (Saldaña et al., 2012) and improvement of the diffusion coefficient for the extraction of phenolics and carbohydrates from the substrate (Kim and Mazza, 2007). Different studies on deffated rice bran reported that the solubility of phenolic acids, protein and carbohydrates increased when the extraction

temperature increased from 50 to 250°C (Hata et al., 2008; Fabian et al., 2010). Furthermore, with the increase in temperature from 25 to 150°C with the accelerated solvent extraction, the diffusion rate of petroleum hydrocarbons, polycyclic aromatic hydrocarbons and polychlorinated biphenyls also increased from 2 to 10 fold, yielding to higher extraction rate and efficiency (Richter et al., 1996).

The removal of high amounts of phenolics can also be attributed to extraction of bound phenolics as the matrix is disrupted at subcritical conditions (Richer et al., 1996). The bound phenolics are ester-linked to the cell-wall polymers (Bonoli et al., 2004), *p*-coumaric acid and ferulic acid being the major bound phenolic compounds present in barley hull (Dvorakova et al., 2008).

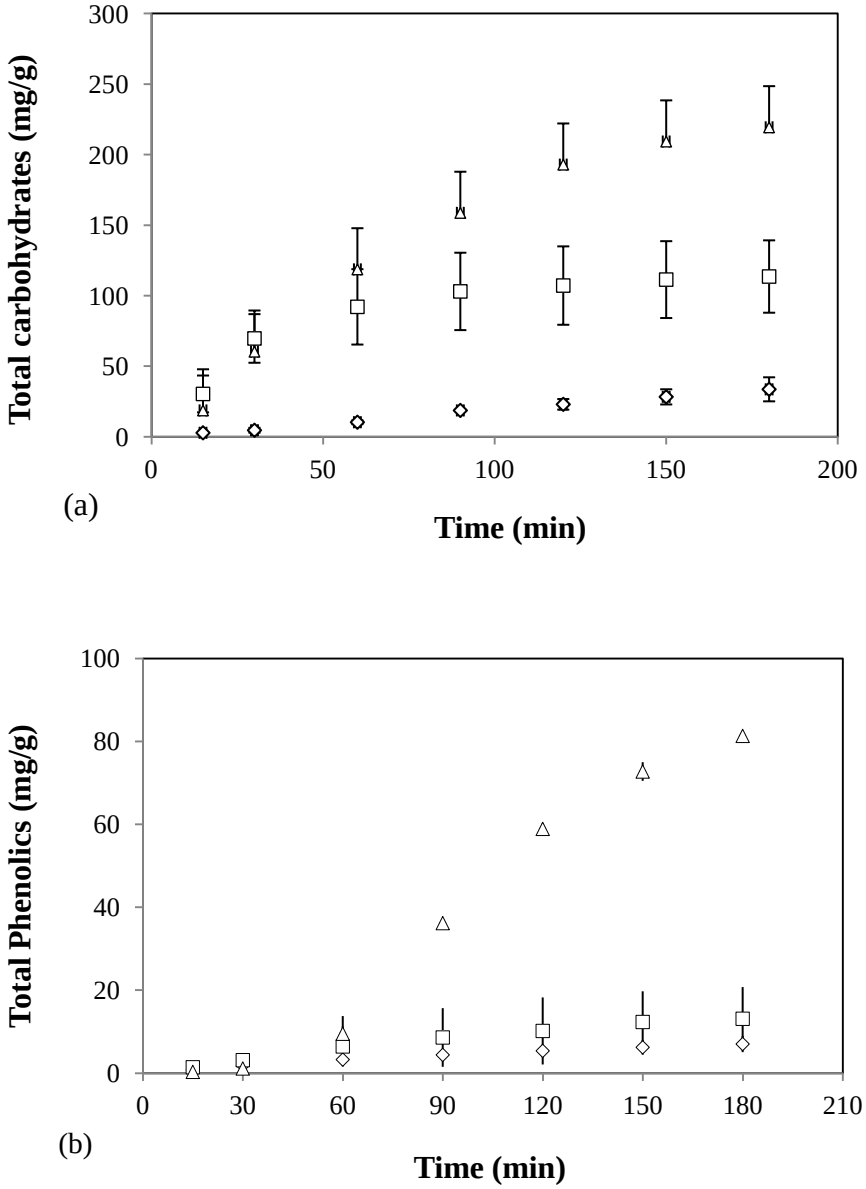


Figure 4.4 Kinetics of (a) total carbohydrates (mg/g), and (b) total phenolics (mg/g) extracted of barley hull at 120°C (◊), 150°C (□) and 180°C (Δ) at 15MPa and 180 min of extraction using SCW extraction**. (**The vertical bars indicate standard error of three replicates, and the standard error is during the total phenolics analysis at 180°C was very small)

To minimize the number of experiments in the preliminary study, a temperature of 150°C for the extraction of total carbohydrates and total phenolics from barley hull was kept constant and the effect of different parameters was investigated**.**

4.3.1.2. Effect of pressure

The effect of pressure at a constant flow rate of 2mL/min and a temperature of 150°C was studied (Fig 4.5 and Appendix C.3). The pressures (5-25MPa) studied for the subcritical water extractions were high enough to maintain the water in the liquid state.

Fig. 4.5 showed that a change of pressure from 5 to 25 MPa had little influence on the extraction of total carbohydrates and phenolics. Wagner and Prub (2002) stated that pressures ranging from 1 to 10MPa at 100 - 300°C are required to maintain water in the liquid state during the subcritical water treatment. Singh and Saldaña (2011) used a pressure of 6MPa at 180°C for the extraction of phenolics from potato peel (81.83mg/100g). In this thesis, approximately 19.7, 18.2, 19.0 and 20.2 mg of total phenolics/g of barley hull were extracted at 5, 10, 15 and 25 MPa, respectively. Therefore, further experiments were only performed at 15MPa. An earlier study on sugarcane bagasse and wood chips showed the highest efficiency (93.1% for wood chips and 88.4% for sugarcane bagasse) at 16MPa and a temperature of 190°C for the extraction of lignin using ethanol+water mixtures (Pasquini et al., 2005). Pasquini et al (2005) concluded that pressure did not play a crucial role on the delignification of wood as the temperature changed from 142-198°C.

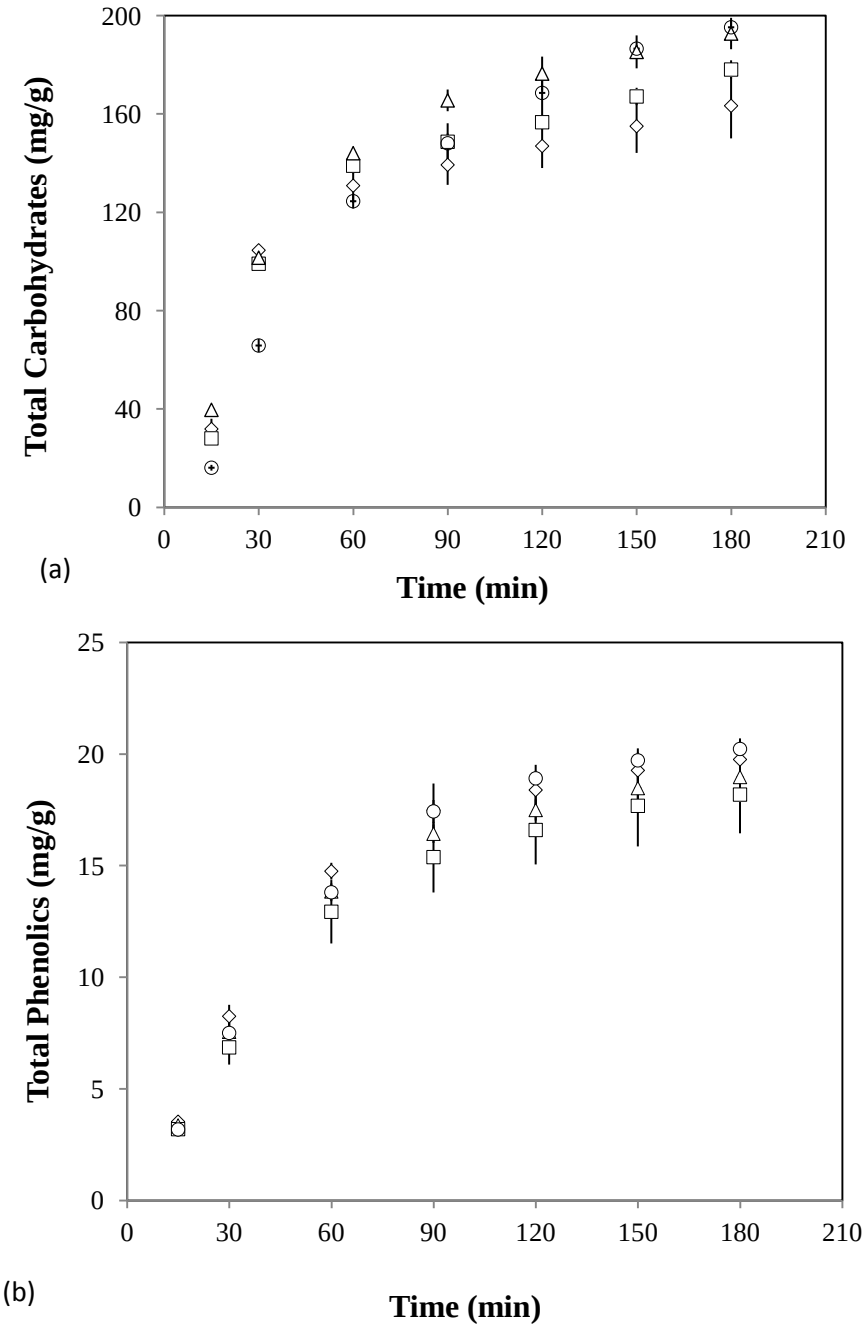


Figure 4.5: Kinetic of (a) total carbohydrates (mg/g), and (b) total phenolics (mg/g) extracted from barley hull at 5MPa (◊), 10MPa (□), 15MPa (Δ) and 25MPa (O) pressures and a temperature of 150°C using SCW extraction**.**

4.3.1.3. Effect of static holding time/residence time

Fig. 4.6(a) and Appendix C.5 showed that the static time had an impact on the extraction of total carbohydrates from barley hull. Yuksel et al. (2011) showed the effectiveness of higher residence time on production of lactic acid from glycerol. After 90 min of residence time at 280°C and 10MPa using 50mM NaOH concentration under subcritical condition, there was a 34.7% increase in the formation of lactic acid from glycerol. The same effect was reported by Pouarli et al. (2010) using subcritical water for the red pine wood cellulose decomposition at 270°C. The high residence time (~30 min) produced more organic acids or by-products from decomposition of glucose or hydroxylmethyl furfural (HMF). However, Fig 4.6(b) and Appendix C.5 showed little impact on the extraction of phenolics with the increase in the static holding time. This result is not in agreement with the study of Pourali et al. (2010), who used a temperature of 220°C and a residence time of 10-20 min, increasing the extraction of total phenolics to about 40% from rice bran biomass. Pourali et al. (2010) concluded that high temperatures with high residence time allow the maximum extraction of phenolics. Thus, an appropriate static holding time during the extraction could provide complete soaking of the matrix and dissolution of target solutes from the surface of the matrix, maximising the extraction of total carbohydrates.

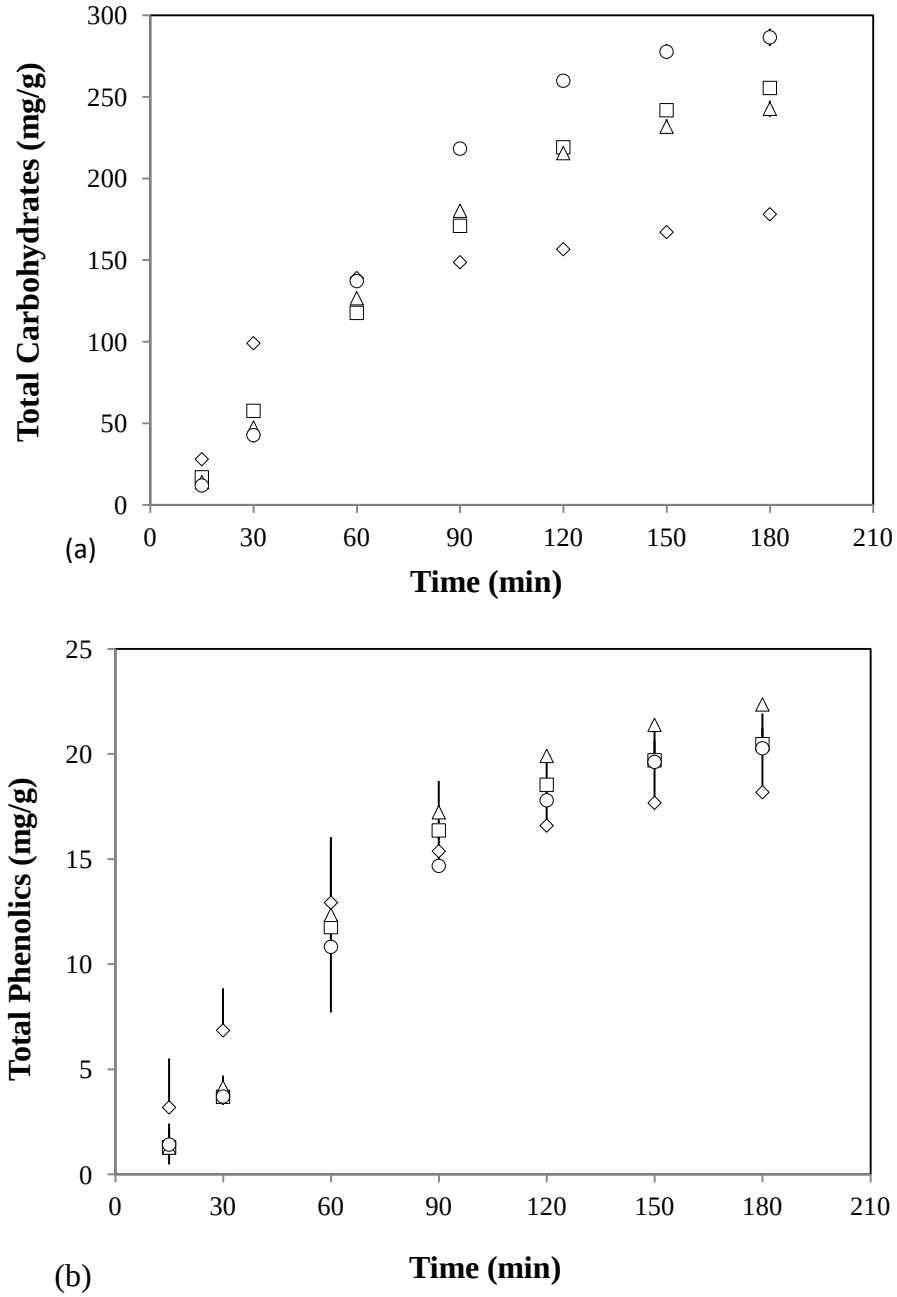


Figure 4.6: Kinetic of (a) total carbohydrates (mg/g), and (b) total phenolics (mg/g) extracted from barley hull at static time of 10 min (◊), 20 min (□), 30 min (**Δ**) and 60 min (**O**) at 15MPa and 150°C using SCW extraction**.**

Based on these results, a holding time of 10 min was adopted for further extractions to evaluate the effect of particle size.

4.3.1.4.Effect of milling of the barley hull

Fig. 4.7 and showed the comparison of total carbohydrates and total phenolics for different extent of milled samples. The barley hull was milled to reduce the particle size and then a study was done on the extent of extraction. The experiments were carried out at a constant temperature of 150°C and a pressure of 15MPa (Fig. 4.7 and Appendix C.7). There was no significant difference in the total carbohydrates and phenolics for different extent of the milled barley hull samples. The main reason of no variation in the extraction of total carbohydrates and phenolics was due to similar average particle size of milled once (0.402 mm), milled twice (0.399 mm) and milled three times (0.377 mm) of barley hull. The determination of the particle size of the three samples were conducted only once and showed no significant difference.

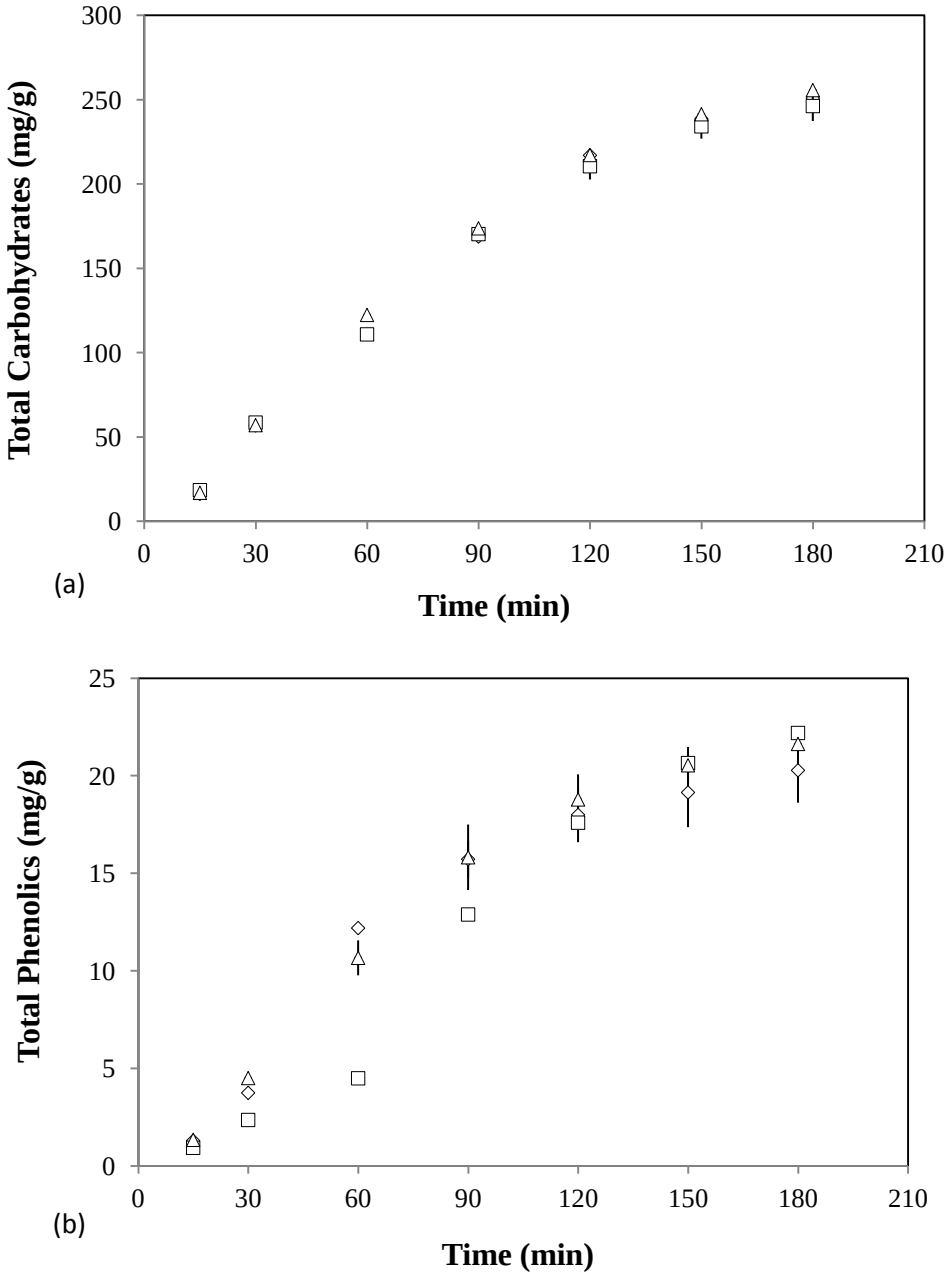


Figure 4.7. Extraction of (a) total carbohydrates (mg/g), and (b) total phenolics (mg/g) of barley hull when milled once (◊), milled twice (□), and milled thrice (Δ) at 15MPa and 150°C using SCW extraction**.**

4.3.1.5. ANOVA analysis

To estimate the significant variables for further studies, the responses of the different parameters were evaluated using ANOVA analysis (Table 4.4 and Appendix C). Table 4.4 showed that the increase in the temperature to 180°C increased significantly the amounts of total carbohydrates and phenolics, which proves that temperature was a significant parameter ($p \leq 0.05$) during the extraction of carbohydrates and phenolics. Thus, temperature was considered for further experiments.

The ANOVA analysis (Table 4.4) also showed that a change of pressure from 5 to 25MPa is not significant on the extraction of total carbohydrates and phenolics. This study shows that only for the first 30 min of the extraction, the static holding time had a significant influence ($p \leq 0.05$) on the extraction of total phenolics. The static holding time during the subcritical water extraction had a greater influence on the total carbohydrates extraction. Furthermore, on the basis of ANOVA analysis, it was observed that there was no significant difference in the total carbohydrates and phenolics using subcritical water extraction for different millings of barley hull.

Table 4.4 ANOVA for the experiments of the preliminary study

Factor:	**Milling**	**Pressure**	**SHT**	**Temperature**
Groups:	**1, 2, 3 (times)**	**5, 10, 15, 20 (MPa)**	**10, 20, 30, 60 (min)**	**120, 150, 180 (^{o}C)**
Dependent var (min)	***p* value**	***p* value**	***p* value**	***p* value**
Total Carbohydrates				
15	0.6498	*0.0110*	*0.0265*	*0.0137*
30	0.8892	*0.0003*	*0.0003*	*0.0005*
60	0.0732	0.0969	*0.0135*	*0.0004*
90	0.2804	0.1614	*0.0001*	*0.0004*
120	0.5940	0.1520	*0.0000*	*0.0016*
150	0.5672	0.0721	*0.0001*	*0.0026*
180	0.5120	0.1221	*0.0002*	*0.0035*
Total Phenolics				
15	0.1441	0.5175	*0.0006*	*0.0205*
30	*0.0164*	0.2658	*0.0311*	*0.0322*
60	*0.0039*	0.1617	0.4517	*0.2658*
90	0.2609	0.3030	0.3060	*0.0368*
120	0.7587	0.3142	0.1989	*0.0022*
150	0.6396	0.4872	0.2702	*0.0004*
180	0.5226	0.4723	0.2344	*0.0002*

4.3.2. Response Surface Methodology (RSM)

Based on the results obtained in the screening study (discussed in section 4.3.1), RSM was used to evaluate the effect of temperature (120-180°C), static holding time (2-20 min), flow rate (2-6mL/min), and ethanol concentration (0-20%) to optimize the extraction of total carbohydrates and phenolics (shown in Table 3.2). Table 3.2 showed that the temperature (experimental/theoretical) changed less than 4% from the beginning to the end of the experiment. This change can influence the reliability of the results when using a statistical method without a duplicate, such as the RSM. But, due to the low standard deviation observed at the center point, the experimental data was used for further experimental interpretations.

4.3.2.1. *Characterization of the subcritical fluid extracts*

All the responses measured during the solid-liquid extraction (discussed in Table 4.2) were studied for the subcritical fluid extraction. The responses for all the different experiments are reported in Table 4.5. As the temperature increased, the pH value of the extract solutions decreased. For example, the extract solution collected at 120°C was neutral (pH of 7.12) while the extract collected at 180°C was acidic (pH of 3.86). This decrease in the pH value may be due to the presence of phenolic compounds (Vermerris et al., 2007), or due to the formation of other compounds, such as organic acids and amino acids as a result of decomposition of barley hull at high temperatures. Pourali et al. (2009) also reported a decrease of pH due the formation of organic acids, such as acetic, formic, glycolic and

levulinic acid from the decomposition of rice bran using subcritical water treatment at 190 to 240°C at the saturation pressure for 5 min.

Table 4.5 also shows the conductivity of the aqueous solution (**κ**), measured after the subcritical fluid extraction. Electrical conductivity of aqueous solution depends on the amount of ions within the solution (Prinkopsky et al., 2007). With the increase in temperature from 120 to 180°C, the conductivity showed an appreciable increase from 841.8 to 2268μs/cm, respectively. Lowering the pH of the extract may contribute to the change in conductivity, dissolution of minerals, and formation of other ions and organic acids (Pourali et al., 2010).

The difference in color of the extracts due to variation of the processing conditions during the sCF extraction was observed using a Hunter *L,a,b* colorimeter. The *L*, *a*, and *b* values showed that the temperature influenced the dark color formation in the extracts (Table 4.5) due to the carbohydrates reaction (Maillard reaction) with amino acids (Pritchard and Adam, 1994; He al., 2012) and also due to the oxidative polymerization of the phenolics (Adams et al., 2004; Fabian et al., 2010). The low value of *L* and high values of *a* and *b* at 180°C indicate that the extract at a high temperature is darker in color (Papadakis et al., 2000). When a temperature above 165°C was used for the extractions, the extracts collected had a dark brown color with a high positive value of *a* (1.78-12.5) as well as *b* (16.83-30.74) as measured using the colorimeter (Table 4.5).

The browning color measured at 294 and 420nm reported in Table 4.5 can be related to the reaction of carbohydrates and proteins (Ogihara et al., 2008), but it is not related to phenolics degradation as phenolics are thermostable at

temperatures over 147°C as reported by Santos et al. (2012). Santos et al. (2012) reported a thermal stability at 199, 213, 147 and 170°C for α-tocopherol, gallic acid, ferulic acid, and caffeic acid, respectively. The change in the absorbance under different temperatures indicates the formation of intermediate and final products in the Maillard reaction (Plaza et al., 2010). In this study, the high values of absorbance (~500 mL/g at 294 nm and ~461 mL/g at 420nm) were achieved at high temperatures (135-180°C) and high concentration of carbohydrates (213.1-489.5 mg/g). These results agree with the study of He et al. (2012) for the extraction of phenolic compounds from the pomegranate seed residues. They reported that the treatment of pomegranate seed residues at 220°C and 6MPa had the highest formation of intermediate products (A280 nm) of the Maillard reaction. So, their observation was that with increasing temperature, the browning intensities of all extracts increased significantly. The same was observed in this study as the high value of the absorbance at 294nm was obtained in 23 (experiment that has the maximum amount of carbohydrates, phenolics, and FRAP). Also, the values of the Hunter *L, a, b* parameters have a linear trend with the values of FRAP. Therefore, it can be concluded that the color of the sample has a direct relation with its antioxidant activity.

The scavenging activity of the DPPH in the extract solutions obtained with the sCF extraction (20.7 to 81.9%) was much higher than the solid-liquid batch extracts (1.0 to 29.7%). The temperature had a significant effect on the scavenging effect. The rise in temperature from 120 to 165°C and 15MPa after approximately 30 min, showed a 60% increase in the antioxidant activity. In

general, phenolic compounds have high antioxidant activity (Parr and Bolwell, 2000). A study of Pourali et al. (2010) reported that the antioxidant activity using DPPH analysis of rice bran extract collected from subcritical water treatment had the same shape profile as the total phenolics of the extract. Extracts obtained during this study at 165°C after 30 min of extraction showed higher antioxidant activity (81.9%) than extracts at 180°C for 25 min (46.2%). The 35% decrease in the antioxidant activity may be due to the short extraction time (25min). Seo et al. (2010) reported that temperature (50-300°C) and time (10-60 min) using subcritical water extraction had a positive effect on the scavenging activity expressed by DPPH value. Subcritical water extracts of Chaga mushroom reported higher antioxidant activity (67.3%) after 60 min of extraction at 200°C than at 150°C treatment for 60 min. The use of 150°C was independent of the extraction time, resulting in the same antioxidant activity after 30 min of extraction. In this study, the extraction was performed for approximately 20 min, resulting in low scavenging activity.

The increase in temperature also had a positive effect on the FRAP antioxidant activity. Treatment of barley hull during this study at 180°C for 25 min resulted in maximum phenolics and thus the highest FRAP value (426mMol ferrous sulphate reduction/g of barley hull), while treatment at 120°C for 25 min resulted in a low antioxidant activity (54 mMol ferrous sulphate reduction/g of barley hull) (Table 4.5).

Table 4.5 Response values for the sCF (aqueous ethanol) extraction

Solvent					Extract solution											
Run	Experimental				Physical properties								Total		Antioxidant activity	
	T**(°C)	Ethanol (%)	SHT** (min)	FR** (mL/min)	pH	RI	k** (µs/cm)	294nm (mL/g)	420nm (mL/g)	*L*	*a*	*b*	CHO** (mg/g)	Phe** (mg/g)	DPPH %	FRAP (mMol FS/g)
1	150	10	2	4	6.98	1.34417	1251	86.8	10.7	95.26	-0.75	7.94	209.85	28.00	20.7	37.2
2	135	15	16	3	6.53	1.33389	1061	500.1	23.7	93.58	-1.12	13.6	213.1	24.34	44.4	161.1
3	165	15	7	3	5.07	1.33864	1946	499.2	94.3	84.25	1.78	26.75	451.65	46.01	75.8	315.6
4	165	15	16	3	5.26	1.33843	1588	497.6	261.9	68.83	9.77	28.85	352.33	45.74	80.4	412.9
5	150	20	11	4	6.12	1.33466	1473	497.4	51.1	90.01	-0.09	17.55	252.93	43.25	43.9	104.3
6	135	15	7	3	7.45	1.33485	889.1	499.1	41.3	87.16	0.55	24.11	194.96	22.59	64.6	293.3
7	165	5	7	3	5.41	1.34851	1747	497.1	27.0	92.64	-1.34	16.83	373.36	31.91	53.1	196.1
8	165	15	16	5	5.11	1.33895	1564	496.5	100.1	81.14	3.21	30.74	398.64	44.74	78.6	342.6
9	150	0	11	4	6.48	1.33382	1432	495.5	40.6	87.13	0.53	20.75	238.58	37.48	60.3	254.7
10	135	5	7	3	7.1	1.33345	1011	253.6	43.2	92.2	-1.39	16.33	194.75	12.87	36.9	83.3
11	135	15	16	5	5.16	1.33862	967.6	498.3	87.5	83.52	2.14	27.02	224.25	35.72	66.2	209
12	165	15	7	5	5.71	1.33835	1766	497.6	143.0	79.15	4.45	30.75	334.79	43.3	80.1	371.8
13	165	5	16	3	4.78	1.34015	1478	497.0	176.7	78.19	4.51	29.69	376.77	32.14	81.9	326.2
14	135	15	7	5	6.6	1.33915	897.4	314.0	53.8	91.25	-1.07	15.66	86.16	13.63	40.0	91.5
15	165	5	16	5	5.36	1.33409	1789	497.9	128.4	81.71	2.87	28.88	397.41	27.39	78.6	333
16	150	10	20	4	5.18	1.34005	1566	496.1	104.1	80.12	4.48	29.55	384.19	45.42	77.5	286
17*	150	10	11	4	5.69	1.33309	1574	497.8	110.5	83.8	2.46	27.89	422.38	45.39	77.8	304.7
18	135	5	7	5	6.95	1.33378	997.1	498.5	94.3	86.46	1.14	23.65	207.84	11.74	58.5	188.9
19*	150	10	11	4	5.84	1.33496	1498	496.9	52.0	88.78	-0.04	24.76	430.02*	46.26	73.4	267.1
20	165	5	7	5	4.95	1.34568	1484	498.3	83.9	86.42	0.81	24.78	334.28	28.37	73.9	269.5
21	120	10	11	4	7.12	1.33332	841.8	133.0	24.5	95.23	-2.87	-13.73	110.57	10.81	25.9	53.8
22	150	10	11	6	6.25	1.33215	1394	495.6	134.3	80.06	3.74	26.82	398.43	48.27	75.8	283.9
23	180	10	11	4	3.86	1.34951	2268	497.6	461.5	53.51	12.5	11.04	489.53	74.84	46.2	425.5
24	135	5	16	3	5.17	1.33312	991.1	498.7	87.4	83.47	2.51	27.67	180.1	13.26	66.1	202.8
25*	150	10	11	4	5.63	1.33496	1429	495.7	125.9	81.25	2.99	27.33	428.13	43.32	76.2	282.7
26	150	10	11	2	5.92	1.33956	1561	499.3	109.4	84.87	1.34	25.17	412.97	39.56	77.8	306.4
27	135	5	16	5	6.35	1.33398	1011	211.1	35.8	93.7	-1.15	12.9	194.83	14.51	31.5	68.1

Table 4.5 continued

Run	Solvent				Extracted solution											
	Experimental				Physical properties								Total		Antioxidant activity	
	T**(°C)	Ethanol (%)	SHT** (min)	FR** (mL/min)	pH	RI**	k** (µs/cm)	294nm (mL/g)	420nm (mL/g)	*L*	*a*	*b*	CHO** (mg/g)	Phe** (mg/g)	DPPH %	FRAP (mMol FS/g)
28*	150	10	11	4	5.39	1.33356	1498	498.5	86.4	86.84	0.35	25.88	428.89	45.59	6.1	269.5
29*	150	10	11	4	5.98	1.33389	1432	496.9	45.5	88.4	0.29	23.67	410.09	45.98	67.3	273.2
30*	150	10	11	4	6.01	1.33822	1399	496.9	98.0	83.1	2.63	29.16	428.74	45.31	78.6	271.9

**T: temperature, SHT: Static holding time, FR: Flow rate, RI: refractive index, κ: conductivity, CHO: carbohydrates, Phe: phenolics, DPPH: 2,2-DiPhenyl-1-PicrylHydrazyl, FRAP: Ferric Reducing/Antioxidant Power, FS: Fe_2SO_4

*Center point, mean ± uncertainty: pH= 5.75±0.126; RI= 1.33478±4.214; κ= 1471.7±0.544; color absorbance at 294nm= 497.11±2.114, color absorbance at 420nm= 86.3±0.155; Hunter LAB: L=84.11±0.668, a=2.44±1.498, b=26.11±1.122; Carbohydrates (mg/g) = 424.71±7.65; Phenolics (mg/g) = 45.30±1.04; DPPH=74.11±7.44, FRAP= 270.66±1.145.

4.3.2.2. *ANOVA analysis for the RSM of subcritical fluid extraction*

Table 4.6 shows the effect of the different variables, such as A (temperature), B (static holding time), C (flow rate) and D (ethanol concentration) on the analyzed parameters. As shown by the ANOVA analysis, the temperature was the most significant factor for the maximum extraction of total carbohydrates and phenolics as well as had a positive influence on the antioxidant activity. In addition to the temperature, the static holding time had a positive effect on the change of pH (p=0.03) and RI (p=0.02) of the extract as well as the extraction of total carbohydrates (p=0.01). As already shown during the solid-liquid extraction the ethanol concentration had a significant impact along with temperature for the extraction of phenolics. On the other hand, the p value of 0.21 showed that the static holding time had less effect on the extraction of phenolics while proved to be effective for the increase the antioxidant activity.

Data obtained for total carbohydrates using Design Expert^{TM} fitted well (R^2 = 0.945) into the quadratic polynomial equation (Equation 4.3) while data for total phenolics fitted well (R^2 = 0.618) to the linear polynomial equation (Equation 4.4). A, B and C are the variables indicated in Table 4.6

$$\text{Total carbohydrates (mg/g)} = 424.71 + 95.05A + 21.18B - 7.83C + 1.05D - 36.15A^2 - 36.19B^2 - 9.74C^2 - 49.73D^2 - 6.09AB - 1.20AC + 7.16AD + 2.53BC + 5.12BD - 11.10CD \quad (p = 0.0001) \quad (4.3)$$

$$\text{Total phenolics (mg/g)} = 34.93 + 11.63A + 2.59B + 0.33C + 4.81D \quad (p = 0.0001) \quad (4.4)$$

Table 4.6 ANOVA analysis for sCF extraction

Response:	**pH**		**RI****		**K****		**420nm**		**CHO****		**Phe****		**FRAP****	
Model:	***quadratic***	***p***	***quadratic***	***p***	***quadratic***	***p***	***quadratic***	***p***	***quadratic***	***p***	***linear***	***p***	***linear***	***p***
Constant	5.757		1.335		1471.667		86.383		424.710		34.925		242.886	
A	*-0.674*	*0.04*	*0.003*	*0.01*	*349.546*	*0.04*	*59.263*	*0.00*	*95.050*	*0.01*	*11.625*	*0.04*	*83.880*	*0.05*
B	*-0.380*	*0.03*	*-0.001*	*0.02*	14.254	0.63	*21.146*	*0.09*	*21.180*	*0.01*	2.594	0.21	*30.970*	*0.04*
C	0.003	0.97	-0.001	0.27	-23.713	0.43	0.879	0.94	-7.829	0.31	0.331	0.87	-6.740	0.64
D	0.004	0.96	0.000	0.99	10.538	0.72	6.246	0.61	1.052	0.89	*4.809*	*0.02*	9.540	0.51
A^2	-0.096	0.25	0.002	*<0.05*	-4.049	0.88	35.091	*0.01*	-36.153	*<0.05*				
B^2	0.051	0.53	0.002	*<0.05*	-40.649	0.16	-11.309	0.32	-36.911	*<0.05*				
C^2	0.052	0.52	0.000	0.73	-23.399	0.40	4.803	0.67	-9.741	0.18				
D^2	0.106	0.20	0.000	0.59	-29.649	0.29	-14.197	0.22	-49.727	*< 0.05*				
AB	0.266	*0.02*	-0.001	0.08	-47.506	0.20	19.819	0.19	-6.094	0.52				
AC	0.113	0.30	-0.001	0.06	-4.806	0.89	-11.269	0.45	-1.197	0.89				
AD	0.030	0.78	-0.002	*0.01*	35.069	0.34	14.856	0.32	7.164	0.45				
BC	0.066	0.54	0.000	0.88	41.381	0.26	-22.944	0.14	21.530	0.03				
BD	-0.001	0.99	0.001	0.06	-21.744	0.55	-2.444	0.87	5.118	0.58				
CD	-0.180	0.11	0.001	0.09	-21.444	0.56	-2.806	0.85	-11.100	0.25				

**RI: refractive index, κ: conductivity, CHO: total carbohydrates, Phe: total phenolics, FRAP: Ferric Reducing/Antioxidant Power, FS: Fe_2SO_4

A = Temperature (°C); B = static holding time (min); C = flow rate (mL/min); and D = ethanol concentration (%)

The RSM optimized the different parameters for the extraction and generated the combination of the parameters which predicted the maximum extraction of total carbohydrates and phenolics. The optimal point for the extraction was 180°C, 15 MPa using 12% ethanol concentrations at 5mL/min flow rate. Therefore, extractions in duplicates were conducted at this optimal point and the average of the total carbohydrates and phenolics obtained after 20 min extraction were 450.3±7.83 mg/g and 70.3±1.33 mg/g, respectively. The predicted values for the total carbohydrates (460.0 mg/g) and the total phenolics (61.6 mg/g) were in agreement with the experimental values obtained during this study. The 1% difference in the predicted and the experimental value of the total carbohydrates and phenolics may be due to the experimental errors. Since the optimal temperature for the maximum extraction for total carbohydrates and phenolics was 180°C in Table 4.5, further studies were performed to study the effect of temperature change to improve the extraction efficiency.

4.3.2.3.*Effect of temperature change during subcritical fluid extraction*

As temperature was the most significant parameter observed during the sCF extraction (as discussed earlier in Table 4.5), further studies at higher temperatures were evaluated. Fig. 4.8 showed that temperature up to 240°C had a positive effect on the extraction of total phenolics (122.38±1.304 mg/g of barley hull) and total carbohydrates (589.4±3.902 mg/g of barley hull) after 20 min of extraction. The total carbohydrates (350.08±21.54mg/g of barley hull) and total phenolics (95.19±3.79mg/g of barley hull) at 260°C showed a substantial decrease, possibly due to thermal degradation (Teo et al., 2010, Teo et al., 2008,

Budrat et al., 2009, Kataoka et al., 2008). The dark color of the extract with the increase of temperature was in agreement with total carbohydrates and phenolics extracted (Fig 4.8).

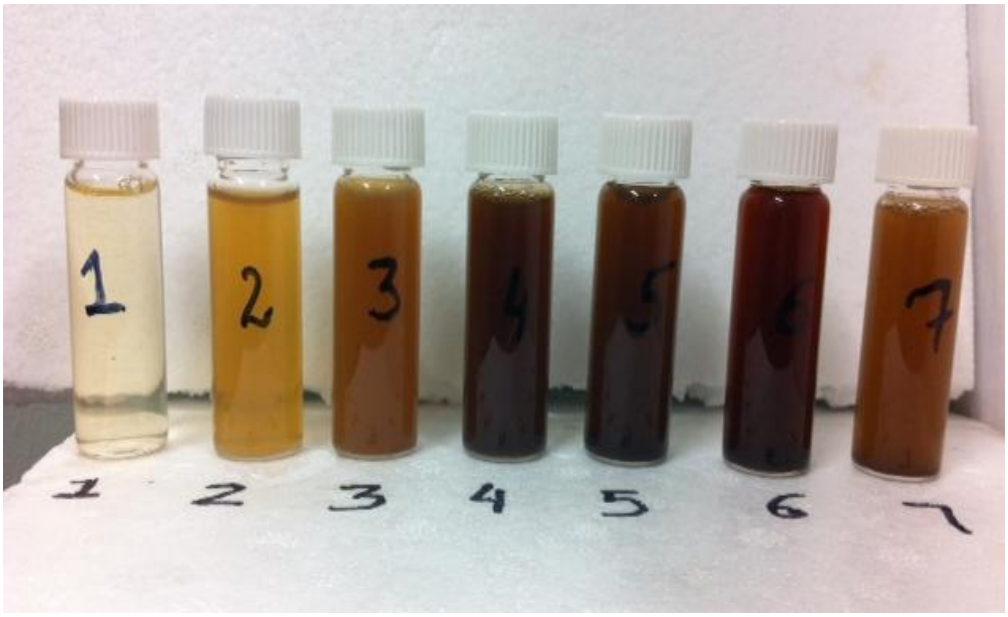
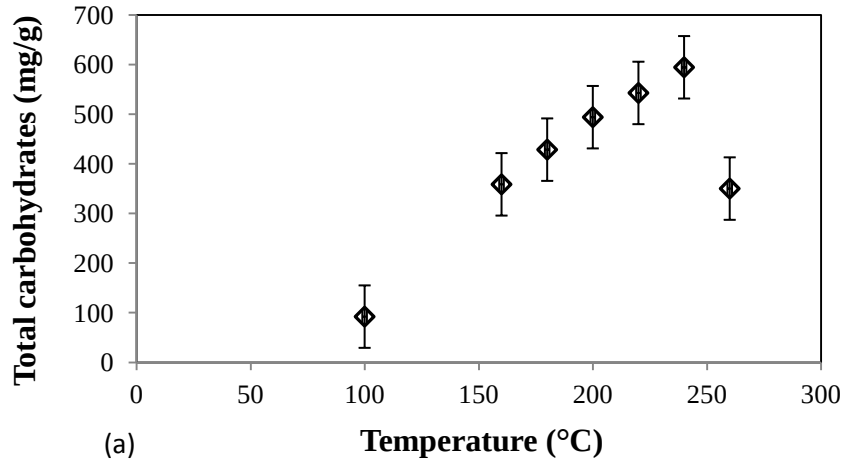
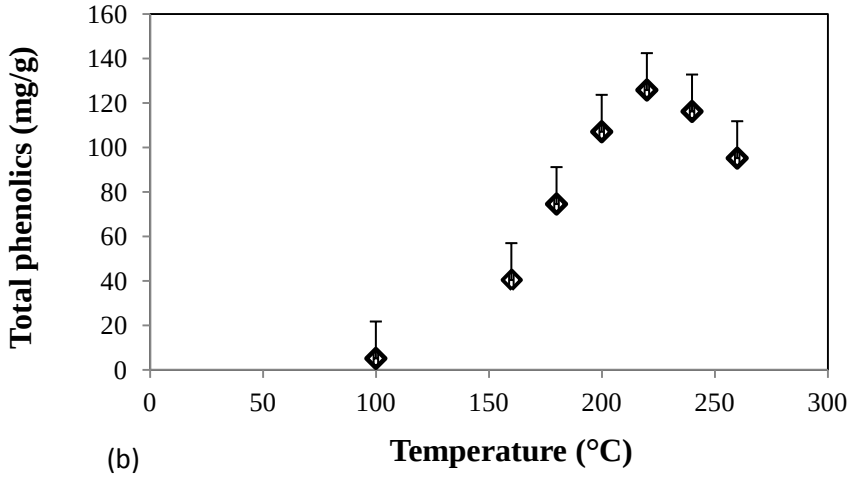


Figure 4.8: Extracts collected at 15MPa using 12% ethanol concentration at a flow rate of 5mL/min and 15 min static holding time and temperatures of 100°C (1), 160°C (2), 180°C (3), 200°C (4), 220°C (5), 240°C (6) and 260°C (7).

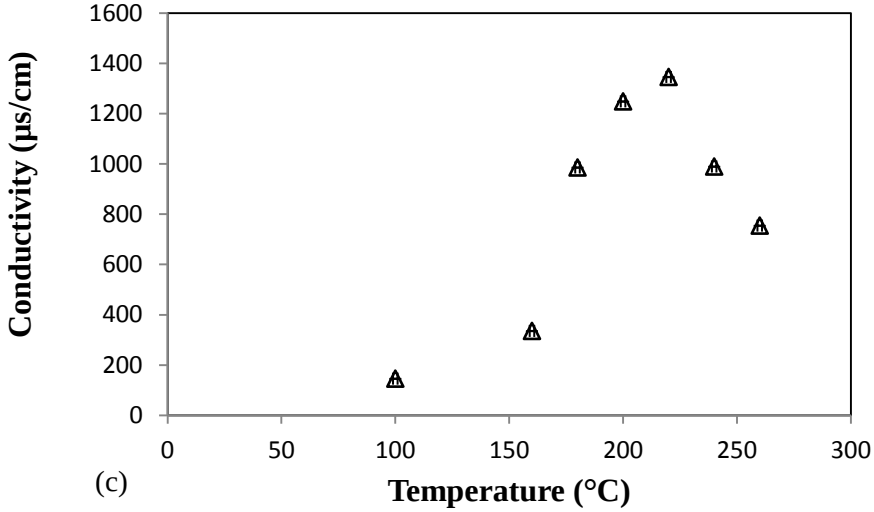
The phenol-sulfuric acid method used for the analysis of total carbohydrates accounts for low molecular weight carbohydrates, such as monosaccharides and disaccharides (Kataoka et al., 2008). Thus, the decrease of total carbohydrates at high temperatures (260°C, Fig 4.9 (a) and Appendix E) can be related to the degradation of carbohydrates into acids, owing to the acidity (pH~2) of the extracts (Pourali et al., 2010). In addition, the extraction at 260°C had a detrimental effect on the extraction of phenolics (97.9mg/g) of barley hull (Fig 4.9 (b) and Appendix E).


Total carbohydrates (mg/g)
700
600
500
400
300
200
100
0
0
50
100
150
200
250
300
Temperature (°C)

(a)


Total phenolics (mg/g)
160
140
120
100
80
60
40
20
0
0
50
100
150
200
250
300
Temperature (°C)

(b)


Conductivity (µs/cm)
1600
1400
1200
1000
800
600
400
200
0
0
50
100
150
200
250
300
Temperature (°C)

(c)

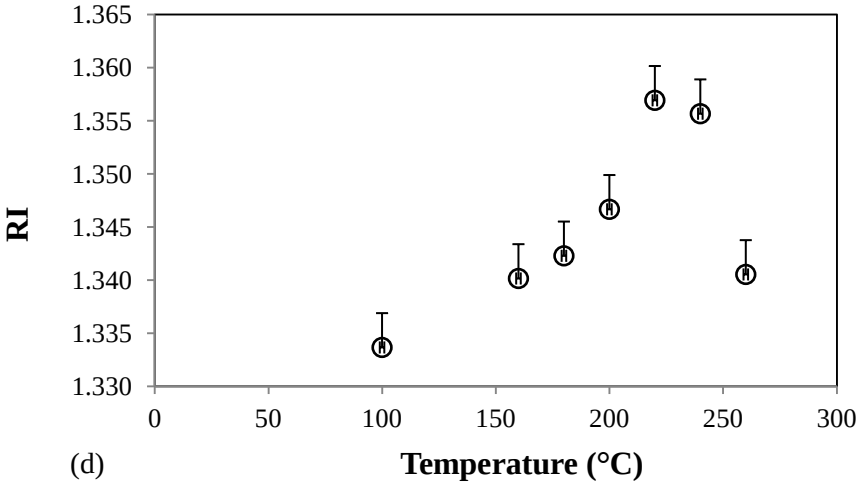


Figure 4.9: Effect of temperature on the (a) extraction of total carbohydrates (mg/g), (b) extraction of total phenolics (mg/g), (c) change in conductivity, and (d) change in RI, at 15MPa using 12% ethanol concentration at 5mL/min, and 15 min of static holding time. Vertical bars indicate standard error of two replicates.

In addition to the total carbohydrates and phenolics analysis, the antioxidant activity was also analysed for the above extracts The change in the antioxidant activity (FRAP analysis) and scavenging activity (DPPH) with the change in temperature from 100 to 260°C is shown in Figure 4.10 (Appendix E). The increase of temperature had a positive impact on the antioxidant activity. It has been observed by Wiboonsirikul et al. (2007) and Hata et al. (2008) that the increase in temperature from 50 to 250°C using subcritical water significantly increased the solubility of antioxidants, proteins and carbohydrates after a 5 min extraction. The same trend was observed for the antioxidant activity of the extracts in this study. The extract obtained at 100°C with 15MPa using 12% aqueous ethanol at 5mL/min had a very low DPPH value (36.97±6.44%) compared to the DPPH of the extract collected at 240°C (89.02±0.40%). The

antioxidant activity determined by FRAP assay for the extract solution collected at 240°C also showed a maximum antioxidant activity in the range of 568.47±1.7mMol ferrous sulphate reduction/g of barley hull.

The trend for the FRAP assay (Figure 4.10) is similar to the trend of total phenolics as observed in Fig 4.9(b). Pourali et al. (2010) reported that phenolics extracted from rice bran possessed antioxidant activity as determined using the DPPH method. Thus, the change in the antioxidant activity can be related to the phenolics in the extracts. Moreover, it has been reported that besides phenolics other non-phenolic compounds, such as carbohydrates and protein derived compounds possess antioxidant activity (Hata et al., 2008). In this study, it was observed that the total carbohydrates extracted at different temperatures (Figure 4.9a) had the same shape profile as the antioxidant activity analysed by the FRAP assay (Fig 4.10). Thus, the increase in the antioxidant activity can also be attributed to the amount of total carbohydrates extracted.

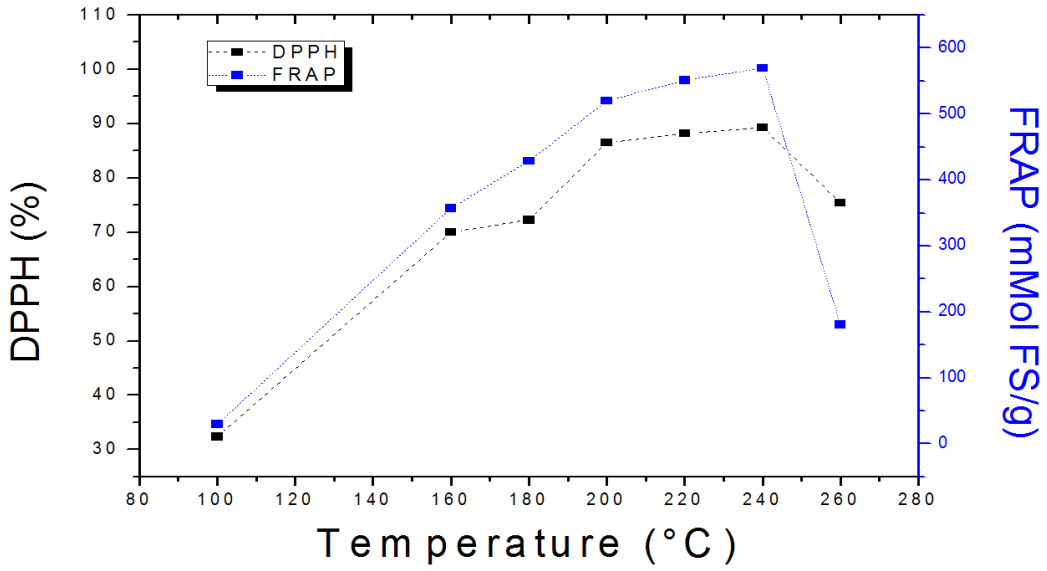


Figure 4.10: Effect of temperature on the FRAP and DPPH values at 15 MPa using 12% ethanol concentration at 5mL/min, and 15 min of static holding time

4.3.2.4. *Kinetics at the optimal point of the sCF*

After determining the optimal point (240°C, 15 MPa using 12% ethanol aqueous solutions at 5mL/min flow rate) to maximize the amount of total carbohydrates and total phenolics extracted, the kinetics of the extraction was obtained. The extract solutions were collected every 5min. As observed in Fig. 4.11 (Appendix G), after 30 min of extraction, there was no significant change in the extraction of total carbohydrates and phenolics. This showed that the extraction time can be reduced to 30 min to obtain the maximum extraction of total carbohydrates (627.3mg/g of barley hull) and less than 20 min can be used for the highest extraction of total phenolics (129.7mg/g of barley hull).

The modeling of the kinetic extraction of total carbohydrates provided the parameters $f_1 = 0.984$, $k_1 = 0.106$, and $k_2 = 0.017$ with a mean square error (mse) of 0.003. The modeling for the kinetic extraction of phenolics provides $f_1 = 0.904$, $k_1 = 0.105$, and $k_2 = 0.016$ with a mse of 0.002. These results show that the mechanism of extraction for total carbohydrates and phenolics are different.

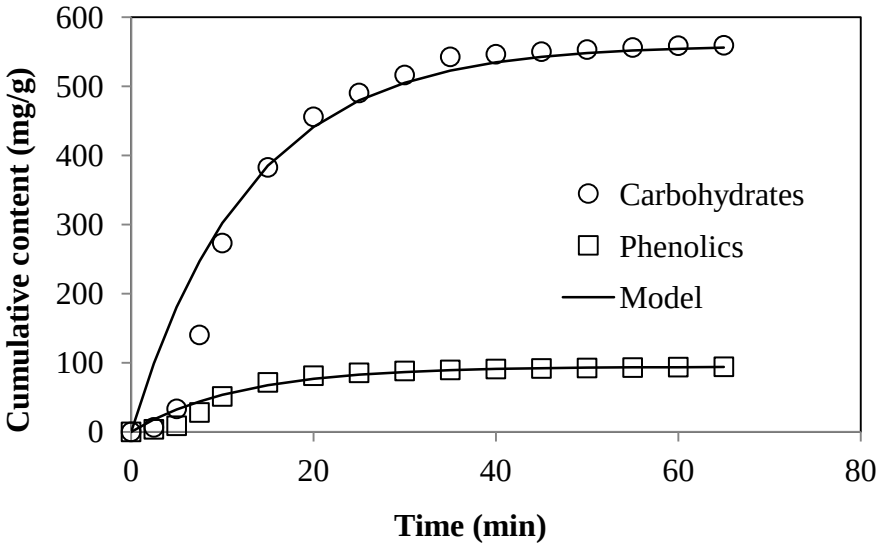


Figure 4.11: Kinetics of total carbohydrates and total phenolics extracted using sCF at 15MPa and 5 mL/min, static holding time of 15 min, ethanol concentration of 12%, and temperatures of 240°C.

Based on these results, the subsequent extractions were conducted at the optimal conditions (240°C, 15MPa, ethanol concentration of 12% flowing at 5 mL/min, and a static holding time of 15 min,) within 32 min to evaluate the effect of pH of the aqueous ethanol solutions.

4.3.2.5. *Study at high and low pH*

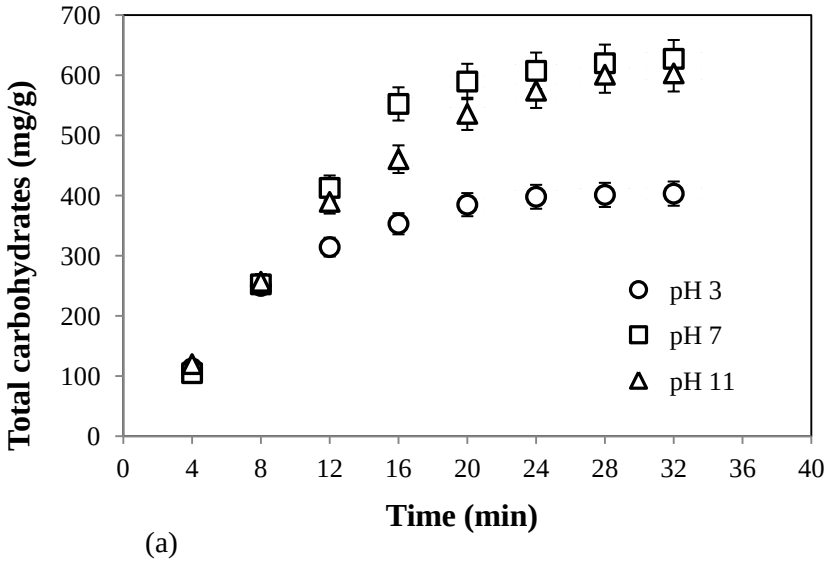
As pH influenced the extraction of total carbohydrates and phenolics using the SLB extraction, the pH effect was studied for the sCF extraction. The ethanol aqueous solvent had a pH of 3 (acidic), 7 (neutral) and 11 (basic), using acetic acid (99%) or sodium hydroxide solution (50%). The experiment was performed at the optimal conditions (240°C, 15 MPa using 12% ethanol concentrations at 5mL/min flow rate) for approximately 32 min of extraction. The influence of pH on the extraction of total phenolics and carbohydrates was observed in Figure 4.12

(Appendix F). The amounts of total carbohydrates and phenolics were 603.13mg/g and 156.13mg/g, respectively, when basic pH was used (pH 11). Similar results for total carbohydrates and total phenolics were obtained using pH 7 or pH 11. There was an appreciable decrease in the extraction of total carbohydrates (403.14mg/g) and total phenolics (125.20mg/g) at acidic pH of 3. Since the pH of the extract solutions changed as reported in Appendix F.3, it is recommended that buffered solutions should be used for further experiments.

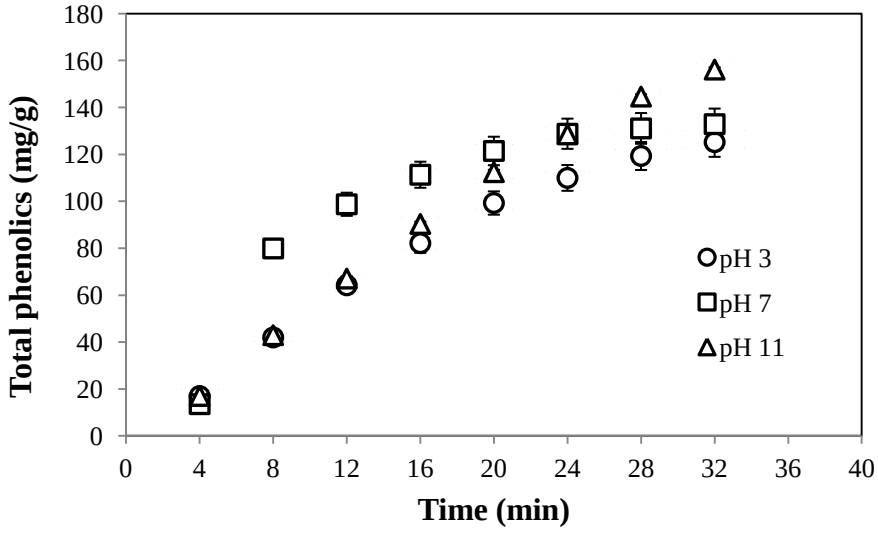
The results of total carbohydrates and total phenolics were in agreement with those reported by Kim and Mazza (2007). They showed that the 0.1M NaOH and the high pH (pH 13) of phosphate buffer (0.01M) at 230°C, 5.2 MPa and a flow rate of 2mL/min were better for the extraction of free phenolics from flax shives than the extraction with water at pH of 6.8. However, the use of phosphate buffer (pH 11) at 230°C and a flow rate of 2mL/min was the best condition for the extraction of total carbohydrates. They did not report the pH of the extract solutions. The high extraction of free phenolics from the matrix can be attributed to the breakage of the C-C bonds, or the ester and ether linkages present in the lignin and carbohydrate-lignin linkages.

The effect of high pH on the extraction of carbohydrates was also discussed by Tumaalii and Wootton (1988). Their findings showed that starch granules swell rapidly in alkaline solvents, resulting in subsequent cell rupture and leaching of enclosed carbohydrates with the increase in temperature (30 to 90°C) of the solvent. Maher (2003) observed a subsequent increase in starch viscosity of various starchy grains, such as barley, corn, wheat, and rye with the

increase of sodium hydroxide concentration from 0.94 to 8.47 mili-equivalents per gram of flour.



(a)



(b)

Figure 4.12: (a) Kinetics of total carbohydrates (mg/g), and (b) total phenolics (mg/g) extracted in 30 min from barley hull at 240°C, 15MPa and ethanol aqueous solutions of pH 3 (o), pH 7 (□) and pH 11 (Δ)

4.4. Extraction using aqueous IL

4.4.1. Solid-IL batch extraction

Table 4.7 shows that little carbohydrates (1.1 - 9.4 mg/g) and high phenolics (15.7 - 39.6mg/g) of barley hull were extracted. The responses for the solid-IL batch extraction using pure m-2-HEAA showed that the IL is specific for the extraction of phenolics (Table 4.7).

Table 4.7 Response for the solid-IL batch extraction

Run	Experimental conditions			Total Carbohydrates (mg/g)	Total Phenolics (mg/g)
	Temperature (°C)	IL:Barley hull (%)	Time (h)		
1	90-100*	30	4	2.73	23.86
2	90-100	10	4	1.12	20.20
3	115-125	30	4	4.34	20.27
4	90-100	50	4	3.50	30.26
5	105-115	42	3	4.80	39.57
6	105-115	18	5	3.69	29.50
7	90-100	30	2	2.79	23.82
8	90-100*	30	4	2.77	25.20
9	75-85	42	5	7.44	33.37
10	90-100	30	6	3.75	15.73
11	90-100*	30	4	2.96	25.42
12	75-85	18	5	3.25	21.95
13	105-115	18	3	5.01	33.17
14	65-75	30	4	2.45	17.97
15	75-85	42	3	2.58	29.66
16	75-85	18	3	3.38	29.48
17	90-100*	30	4	2.96	25.33
18	90-100*	30	4	3.23	27.18
19	105-115	42	5	9.45	37.82
20	90-100*	30	4	3.06	24.71

*Center point, mean ± uncertainty: Carbohydrates (mg/g) = 2.95±1.016; Phenolics (mg/g) = 25.28±0.716.

Since this extraction using m-2-HEAA was conducted for the first time, the spectrophotometric analysis for total carbohydrates quantification needed

further modifications. It was observed that the pure IL was a yellowish color but no absorbance at the wavelengths of 490 nm and 765 nm, were recorded. Thus, the yellow color of the pure IL had no effect on the total carbohydrates and phenolics analysis

The basic pH of the final supernatant (pH of 12) had an effect on the analysis of total carbohydrates. To study the pH effect of the extracts on the analysis, different concentrations of aqueous glucose were prepared in 5g of IL (1, 2 and 3mg/g), and the pH was adjusted to 12 with 0.1mol/L NaOH. Following the protocols described earlier for the total carbohydrates analysis (Section 3.3.1.5), after the addition of the phenol and sulfuric acid to the solutions prepared, the absorbance was read at 490nm. The absorbance showed a decrease with the increase of glucose concentration (Table 4.8). For instance, glucose + IL solution at 2mg/g had an absorbance of 0.260, which decreased to 0.176 with the increase in the concentration to 3mg/g.

Due to this decrease in absorbance, the pH 12 was then adjusted to neutral (pH of 7) using acetic acid to reduce the effect of pH on the analysis. In addition, a blank was used to consider the effect of pH and addition of acetic acid on the analysis. After the neutralization of samples, the absorbance of the different IL solutions showed a linear increase with the increase in the concentration of glucose. The absorbance of 1mg of glucose/g of IL solution was 0.171, which increased to 0.203 with the increase in the concentration to 2mg/g and further increased to 0.264 for the concentration of 3mg/g. Thus, it was concluded that a

neutral pH is required for the analysis of the carbohydrates. Also, it was observed that pH had no influence on the phenolics analysis.

Table 4.8 Absorbance at 490nm recorded at different pH (12 and 7) and glucose concentration (1, 2, and 3 mg/g of pure IL)

Concentration of glucose (mg/g solution)	pH 12			pH 7		
	Glucose (g)	IL added (g)	Abs at 490 nm	Glucose (g)	IL added (g)	Abs at 490 nm
1	0.005	5.001	0.092	0.005	5.052	0.171
2	0.010	5.000	0.260	0.011	5.024	0.203
3	0.015	5.001	0.176	0.015	5.036	0.264

4.4.2. Extraction under subcritical conditions

After observing the effectiveness of using subcritical aqueous ethanol for the extraction of total carbohydrates and phenolics (Section 4.3), the aqueous ethanol solvent was replaced by aqueous solution of m-2-HEAA. The temperatures studied for the extraction were 100 and 160°C. It was observed that the use of 165°C produced a dark brown colored extract. However, Alvarez (2010) reported that the 2-m-HEAA degrades only at 200°C. Therefore, the extraction was carried out at the maximum temperature of 160°C. Since the extract solutions obtained using the sCF (aqueous IL) had pH between 6 or 7 (Table 4.9), further adjustment of the pH was not required for this analysis. The responses of total carbohydrates and total phenolics under different conditions of subcritical aqueous IL extraction are reported in Table 4.9.

Table 4.9 Responses for subcritical water+IL extraction

Sample	Temperature (°C)	Static holding time (min)	IL:water (%v/v)	Total CHO (mg/g)	Total Phenolics (mg/g)	pH of the extract solution
1	100	2	20	11.04	65.83	6.46
2	100	15	50	11.45	106.56	6.78
3	160	2	50	54.18	189.05	6.14
4	160	15	20	30.22	129.73	6.11

For the analysis of total carbohydrates and phenolics, the standard calibration curves were prepared using 20% and 50% (v/v) IL solution at different concentrations of glucose (calibration curve shown in Appendix A.4). The extraction of total carbohydrates and phenolics is influenced by an increase of temperature and the IL:water ratio. When comparing these results with the subcritical ethanol:water extraction (Table 4.4), this extraction was more effective for the removal of total phenolics. For instance, the experiments with IL:water (50%) at 160°C and 2 min static time extracted more phenolics (189.0 mg/g) than the experiments performed with 12% aqueous ethanol at 160°C (40.6 mg/g). Also, with aqueous ethanol at 240°C and pH of 11 (Table 4.4), the phenolics extraction (159.2mg/g) was less than the extraction at 160°C with 50% aqueous IL. However, carbohydrates extraction (54.2mg/g) using pressurized aqueous IL solutions were not as effective as with the subcritical aqueous ethanol (323.1 mg/g) at 160°C. This may be due to limited reaction of cellulose and hemicellulose with the IL. Thus, the use of aqueous IL could be restricted for the extraction of total phenolics but not for carbohydrates. Therefore, the use of subcritical aqueous ethanol was considered to be the best method for an efficient extraction of both total carbohydrates and phenolics.

4.5. SC-CO_2 extraction

The use of SC-CO_2 was considered for the extraction of total carbohydrates and phenolics in this thesis. The experiments were performed for 3h at two different temperatures (60 and 120°C) and all the other factors, such as pressure (30MPa), CO_2 flow rate (1mL/min), ethanol flow rate (2mL/min), and ethanol concentration (50:50% v/v) were kept constant. At 60°C and 30MPa, the amounts of total carbohydrates and total phenolics were 69.0±0.819 and 22.4±0.916 mg/g, respectively, after 3h extraction. As the temperature increased to 120°C at 30 MPa, the amounts extracted increased to 73.4±0.016 and 67.9±0.421 mg/g of total carbohydrates and phenolics, respectively. The increase of temperature also resulted in a significant increase of phenolics but not for total carbohydrates. The increase of phenolics extraction can also be attributed to the co-solvent addition which showed to have a positive effect during the solid-liquid and subcritical extraction. However, the contents of total carbohydrates and phenolics (73.4±0.016 mg/g and 67.9±0.421 mg/g, respectively) removed using SC-CO_2 extraction was very low compared to the use of pressurized fluids (627.3mg carbohydrates/g of barley hull and 129.7mg pehnolics/g of barley hull); therefore, the optimization of the variables was not performed.

Chapter 5: Conclusions and Recommendations

5.1. Conclusions

- Three methods were used to evaluate the extraction of total carbohydrates and total phenolics from barley hull: solid-liquid extraction, sCF/pressurized fluid extraction and SC-CO_2 extraction.

(a) Variables investigated

- Temperature was the most important variable for the solid-liquid extraction and the sCF extraction ($p \leq 0.05$), while the static holding time was also important for the subcritical aqueous ethanol extraction.
- The addition of a polar solvent, such as ethanol to water solutions increased the extraction of total phenolics and total carbohydrates. At 150°C and 15MPa, subcritical water was able to extract 21.8 mg of phenolics/g of hull while 43.25 mg of phenolics/g of barley hull was extracted with the addition of 20% ethanol. Furthermore, pressurized aqueous IL extraction at a concentration of 50% yielded 120 mg of phenolics/g of barley hull at 160°C and 15MPa. In addition, the use of 20% ethanol showed an increase in the total carbohydrates extraction (252.9 mg carbohydrates/g of barley hull) than extraction with subcritical water alone at 150°C and 15MPa (113.5 mg of carbohydrates/g of barley hull).
- Changing the pH of the aqueous solvent from pH 3 to pH 11 resulted in an increase in the extraction of total phenolics and total carbohydrates. At 240°C, 15MPa and 5mL/min, the 12% aqueous ethanol solvent at pH 3 extracted low amounts of total carbohydrates and total phenolics (403.1 mg

of carbohydrates/g of barley hull and 125 mg/g of phenolics/g of barley hull, respectively) than the solvent maintained at pH 11 (603.1 mg of carbohydrates/g of barley hull and 156.1 mg of phenolics/g of barley hull, respectively).

(b) Physico-chemical and functional characteristis of extracts

- The antioxidant activity of the subcritical aqueous ethanol extracts antioxidant activity is related to the brown color formation and the phenolics concentration. This supports the hypothesis of the formation of new antioxidant compounds from Maillard reactions using the sCF extraction.
- The acidic pH (~ pH 3) of the extracts obtained from sCF extraction under optimal extraction conditions (240°C, 15MPa, 15 min static time and 5mL/min) facilitated better extraction of carbohydrates and phenolics.
- RI and conductivity for all the extracts obtained using the different extraction methods increased with the increase of temperature.

(c) Process

- SC-CO_2 (120°C, 3MPa, and 2mL/min) and subcritical IL (160°C, 15MPa and 5mL/min) methods extracted low amounts of carbohydrates (73.4 and 54.2 mg/g, respectively) and high amounts of phenolics (67.9 and 189.04 mg/g, respectively).
- The maximum extraction of total carbohydrates and phenolics was obtained at 240°C, 15MPa, 15 min static time, and 12% ethanol flowing with 5mL/min using subcritical aqueous ethanol (589.4 and 122.38 mg/g,

respectively), while the minimum extraction was obtained at 70°C with 41% aqueous ethanol having pH 8 using solid-liquid batch extraction (13.9 mg/g and 10.2 mg/g, respectively).

- The highest amount of phenolics (189.1mg/g) and low amount of carbohydrates (54.2mg/g) obtained using the subcritical aqueous IL (50%) extraction at 160°C shows potential for separation of phenolics and carbohydrates for future developments.

(d) Modelling

- The accuracy of RSM was found to be affected by the limitations on the number of experiments, noise in the data and inadequacy of the fitting model
- The kinetic model of Kandiah and Spiro (1990) shows that the mechanism of extraction was controlled by the internal diffusion for carbohydrates and by the internal/external elution for phenolics. The modeling fit had a mean square error of 0.003 and 0.002 for carbohydrates and phenolics, respectively.

5.2. Recommendations

The following recommendations are for any further studies derived from this research:

- Further studies are needed to analyze the individual phenolics extracted during the subcritical fluid extraction.
- To study the effect of high temperature on the degradation of carbohydrates.

- Further studies using subcritical IL to improve the sCF process for the extraction of phenolics.
- The utilization of the extract as a beverage in the food industry by studying its sensory properties.
- Scale up of the process.
- Further studies to determine the molecular size of carbohydrates using microscopy techniques.

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APPENDIX A

STANDARD CURVES AND CALCULATIONS

(a) Total carbohydrate content

Total carbohydrates of the extracts were estimated using a modified spectrophotometric method explained in Section 3.3.3.1.5.

Glucose was used as a standard and the reagents were prepared as per the methodology. The glucose standard curves were prepared before the total carbohydrates analysis and are valid only for the batch of experiments analyzed the same day. The calculation was done according to the respective equation of the straight line. Results were calculated on the basis of the standard curves such as the one presented here (refer to Section 3.3.1.5 for more details).

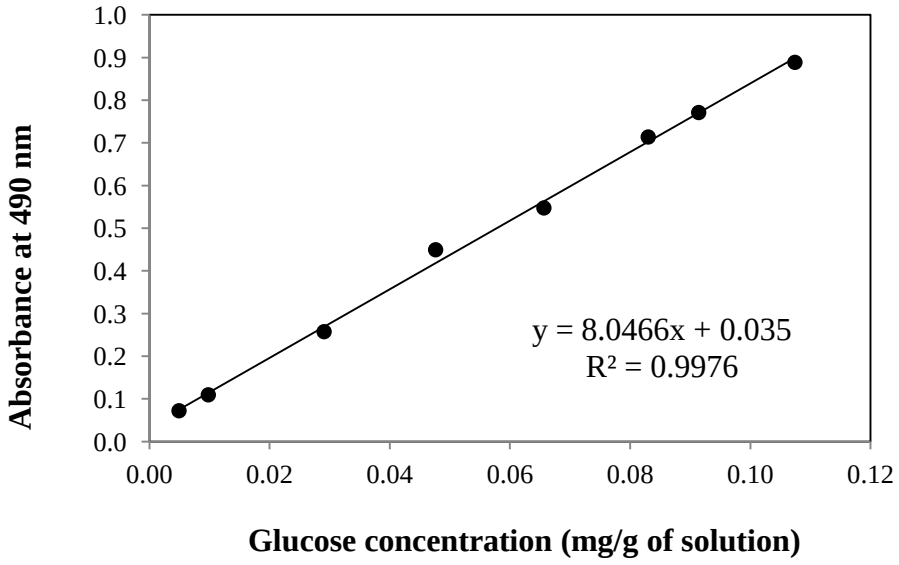

Figure A.1: Standard curve for total carbohydrates content

The content of the sample can be calculated using the equation of the straight line:

$y = 8.0466x + 0.035$

where, *y* is the absorbance of the glucose solution, and *x* is the concentration of the carbohydrates in the sample (or the glucose standard solution).

Sample calculation: For instance,

when, y = 0.105 (Absorbance measured)

then, $0.105 = 8.0466x + 0.035$

therefore, $x = 0.009$ mg glucose equivalent/g of extract

(b) **Total carbohydrates content for IL solution**

Glucose curve for analyzing extract collected using subcritical IL method was prepared using the 20 and 50% of IL as a solution. The calculation for the concentration of total carbohydrates was done the same way as discussed above. The glucose curve using 50% IL is presented here.

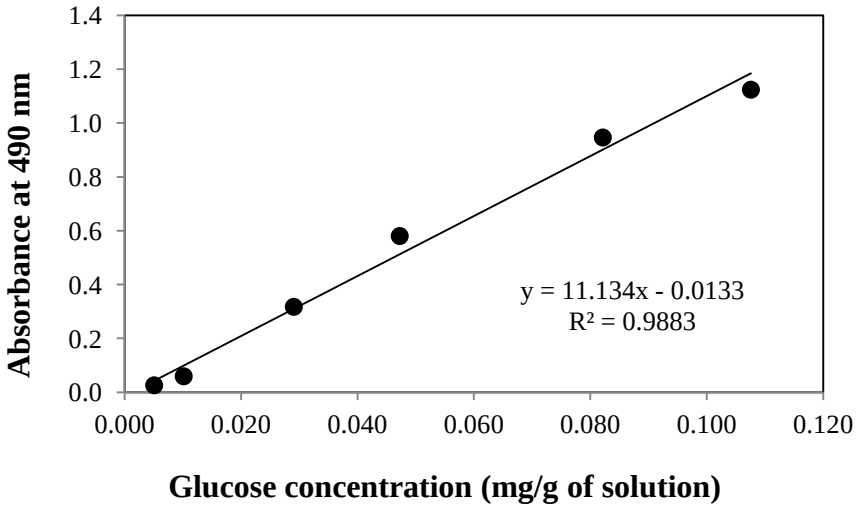

Figure A.2: Standard curve for total carbohydrates content using 50% IL

(c) Total phenolics content

Total phenolics content of the extracts were calculated using a modified spectrophotometric method explained by Singelton and Rossi (1965). Gallic acid was used a standard and the reagents were prepared as discussed in Section 3.3.3.1.6. The calibration curve is valid only for the batch of experiments analyzed the same day. Results were calculated on the basis of a standard curve, such as the one presented here (refer to Section 3.3.1.6 for more details).

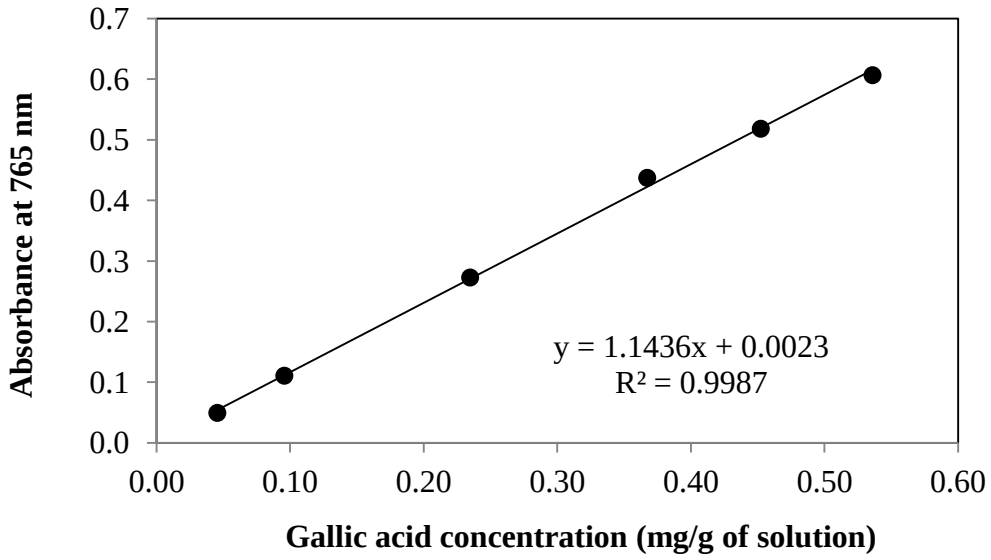

Figure A.3: Standard curve for total phenolics content

The concentration of the targeted compound in the extracts was calculated using the equation of the straight line:

$$y = 1.1436x + 0.0023$$

The sample calculation was done the same way as discussed before for total carbohydrates analysis.

(d) FRAP assay

Ferric reducing antioxidant power of each extract was calculated using ferrous sulphate solutions. The antioxidant activity was expressed in millimoles of ferrous sulphate reduced per gram of extract. The figure below shows one standard curve for the FRAP analysis.

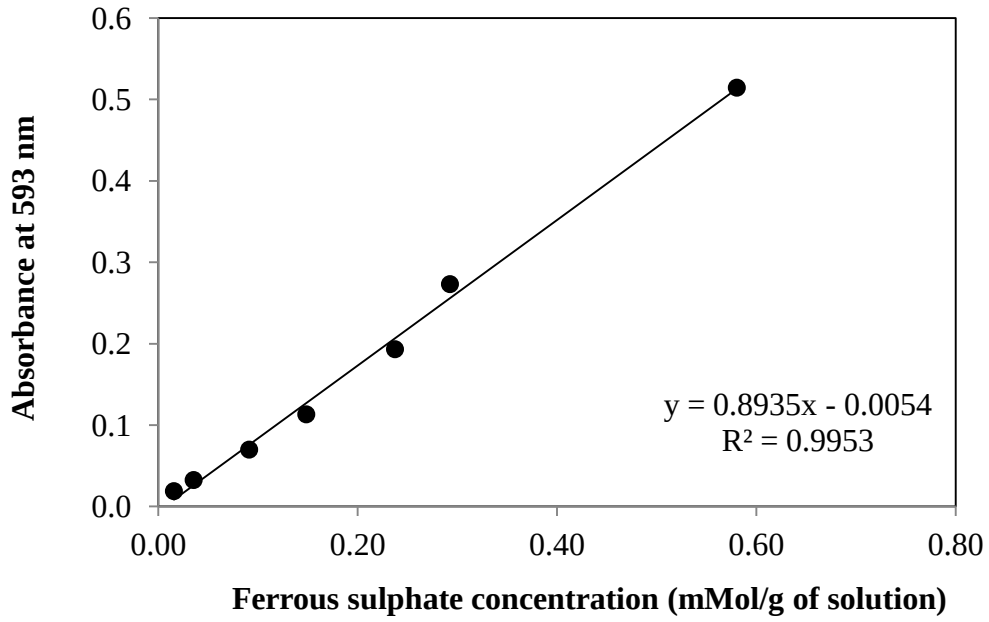

Figure A.4: Standard curve for the FRAP assay

The same type of standard curve was prepared for estimating the equation of the straight line for the calculation of the concentration of the respective compound in the extract. The sample calculation was done as per the above mentioned analysis.

APPENDIX B

SOLID-LIQUID BATCH EXTRACTION: Response Surface Methodology (Refer to Table 4.2)

(a) Total carbohydrates: Statistical analysis was done by Design Expert 6.0.6. The quadratic model was significant for the total carbohydrate analysis.

Table B.1: Experimental and predicted values for the RSM experiments of solid-liquid batch extractions

Run	Total Carbohydrates (mg/g)		
	Actual value	Predicted value	Residual value
1	9.37	10.16	-0.79
2	11.02	10.67	0.34
3	11.46	10.75	0.71
4	9.24	8.85	0.39
5	12.47	11.79	0.68
6	11.81	11.19	0.62
7	8.99	8.20	0.79
8	11.81	10.67	1.14
9	8.85	8.94	-0.09
10	10.78	10.60	0.18
11	9.12	9.47	-0.35
12	9.46	10.67	-1.21
13	12.01	12.49	-0.48
14	10.16	10.67	-0.51
15	13.94	13.14	0.79
16	9.66	10.67	-1.01
17	11.95	11.88	0.07
18	9.47	9.56	-0.09
19	9.91	10.67	-0.76
20	11.99	12.40	-0.42

The plot of actual value versus the predicted value shows that the quadratic model fits with low deviation of the experimental data (data not shown).

(b) Total phenolics content: The linear model was significant. The residual values are shown below.

Table B.2: Experimental and predicted values for the RSM experiments of solid-liquid batch extraction

Run	Total Phenolics (mg/g)		
	Actual value	**Predicted value**	**Residual value**
1	6.46	8.74	-2.28
2	10.41	9.18	1.23
3	10.28	10.07	0.21
4	8.22	7.08	1.14
5	11.64	11.13	0.51
6	14.81	11.92	2.89
7	7.11	7.31	-0.20
8	9.15	9.18	-0.04
9	8.88	7.86	1.02
10	9.64	9.24	0.40
11	7.16	8.40	-1.24
12	9.11	9.18	-0.07
13	10.49	12.07	-1.58
14	8.28	9.18	-0.90
15	12.50	11.69	0.81
16	9.12	9.18	-0.06
17	10.41	9.61	0.80
18	9.96	10.91	-0.95
19	8.93	9.18	-0.25
20	10.41	11.86	-1.45

APPENDIX C

SUBCRITICAL WATER EXTRACTION (sCF): screening experiments

(a) Temperature variation: Total carbohydrates and total phenolics content of the extract solutions were used to analyze the significance of temperature in the SCW extraction.

Table C.1: Total carbohydrates and total phenolics at 15MPa, 2mL/min and 60 min of static holding time

Temperature (°C)	Time (min)	Total Carbohydrates (mg/g)				Total Phenolics (mg/g)			
		1	2	3	Average	1	2	3	Average
120	15	2.73	2.04	3.41	2.73	0.74	0.70	0.81	0.75
	30	3.64	3.44	6.58	4.55	1.55	1.70	2.23	1.83
	60	10.29	8.89	11.61	10.26	2.74	2.92	4.05	3.23
	90	18.75	18.12	18.99	18.62	3.62	3.79	5.67	4.36
	120	20.33	21.20	27.25	22.92	4.32	4.57	7.13	5.34
	150	24.93	25.33	34.45	28.23	5.04	5.29	8.24	6.19
	180	28.75	28.57	43.42	33.58	5.73	5.97	9.24	6.98
150	15	45.14	20.51	25.26	30.30	0.85	1.37	1.90	1.37
	30	88.92	64.32	55.70	69.65	1.91	4.07	3.29	3.09
	60	122.11	83.15	70.90	92.05	3.93	11.98	3.29	6.40
	90	132.48	98.33	78.21	103.01	5.79	16.60	3.30	8.56
	120	137.41	101.26	82.78	107.15	6.99	19.33	4.10	10.14
	150	141.39	104.51	88.22	111.38	7.65	20.84	8.41	12.30
	180	141.95	106.66	92.06	113.55	7.99	21.87	9.38	13.08
180	15	16.07	17.72	21.98	18.59	0.57	0.11	0.13	0.27
	30	58.04	57.26	65.50	60.27	1.47	0.91	0.89	1.09
	60	113.59	121.71	120.56	118.62	13.19	11.87	3.35	9.47
	90	171.41	170.56	134.03	158.67	13.23	47.02	48.22	36.16
	120	218.92	219.21	140.55	192.89	38.09	67.89	70.68	58.89
	150	241.53	242.04	144.24	209.27	53.98	79.85	84.35	72.73
	180	255.54	255.32	147.17	219.34	63.81	89.43	90.71	81.32

Table C.2: ANOVA analysis to evaluate the effect of temperature in the SCW extraction at different time intervals

Time (min)		Total Carbohydrates (mg/g)					Total Phenolics (mg/g)				
		Sum of Squares	df	Mean Square	F	*p*	Sum of Squares	df	Mean Square	F	*p*
15	Between Groups	1149.352	2.000	574.676	9.549	0.014	1.836	2.000	0.918	7.953	0.021
	Within Groups	361.103	6.000	60.184			0.693	6.000	0.115		
	Total	1510.455	8.000				2.529	8.000			
30	Between Groups	7429.322	2.000	3714.661	34.708	0.001	6.139	2.000	3.069	6.428	0.032
	Within Groups	642.162	6.000	107.027			2.865	6.000	0.477		
	Total	8071.484	8.000				9.004	8.000			
60	Between Groups	19135.977	2.000	9567.988	38.988	0.000	58.286	2.000	29.143	1.666	0.266
	Within Groups	1472.440	6.000	245.407			104.971	6.000	17.495		
	Total	20608.417	8.000				163.257	8.000			
90	Between Groups	29831.494	2.000	14915.747	37.034	0.000	1790.088	2.000	895.044	6.022	0.037
	Within Groups	2416.543	6.000	402.757			891.739	6.000	148.623		
	Total	32248.037	8.000				2681.827	8.000			
120	Between Groups	43335.397	2.000	21667.698	22.877	0.002	5266.523	2.000	2633.262	20.042	0.002
	Within Groups	5682.957	6.000	947.160			788.342	6.000	131.390		
	Total	49018.354	8.000				6054.865	8.000			
150	Between Groups	49268.134	2.000	24634.067	18.743	0.003	8115.842	2.000	4057.921	37.268	0.000
	Within Groups	7885.870	6.000	1314.312			653.302	6.000	108.884		
	Total	57154.004	8.000				8769.145	8.000			
180	Between Groups	52093.275	2.000	26046.638	16.851	0.003	10219.393	2.000	5109.696	52.398	0.000
	Within Groups	9274.035	6.000	1545.672			585.097	6.000	97.516		
	Total	61367.310	8.000				10804.489	8.000			

(b) Pressure variation: Effect of pressures on the extraction.

Table C.3: Total carbohydrates and total phenolics for the SCW extraction at different pressures, 150°C, 2mL/min and 60 min static holding time

Pressure (MPa)	Time (min)	Total Carbohydrates (mg/g)			Total Phenolics (mg/g)		
		1	2	Average	1	2	Average
5	15	27.69	36.01	31.85	3.78	3.27	3.52
	30	103.75	105.36	104.56	8.76	7.74	8.25
	60	137.98	123.71	130.84	14.37	15.12	14.75
	90	147.40	131.17	139.29	16.27	18.68	17.47
	120	155.90	138.01	146.95	17.53	19.25	18.39
	150	165.87	144.16	155.02	18.46	20.07	19.26
	180	176.59	150.06	163.32	19.19	20.32	19.75
10	15	25.70	30.36	28.03	3.25	3.12	3.19
	30	101.08	97.07	99.07	6.08	7.62	6.85
	60	135.83	142.07	138.95	11.51	14.35	12.93
	90	145.36	152.04	148.70	13.79	16.96	15.38
	120	153.62	159.64	156.63	15.05	18.14	16.59
	150	163.58	170.66	167.12	15.86	19.50	17.68
	180	174.32	181.83	178.08	16.45	19.92	18.18
15	15	39.24	40.04	39.64	4.11	3.33	3.72
	30	100.29	102.77	101.53	9.24	8.17	8.70
	60	144.39	143.70	144.05	16.27	15.77	16.02
	90	169.94	161.11	165.53	19.08	18.26	18.67
	120	183.34	169.52	176.43	19.91	18.97	19.44
	150	191.95	178.56	185.26	20.61	19.52	20.07
	180	199.11	186.33	192.72	20.93	19.77	20.35
25	15	14.92	17.26	16.09	2.86	3.49	3.18
	30	63.11	68.50	65.81	7.01	8.00	7.50
	60	121.52	127.50	124.51	13.40	14.20	13.80
	90	139.92	156.21	148.07	17.94	16.92	17.43
	120	159.54	177.61	168.57	19.51	18.30	18.91
	150	187.74	185.40	186.57	20.25	19.18	19.71
	180	198.27	192.26	195.26	20.70	19.75	20.23

Table C.4: ANOVA analysis to evaluate the effect of pressure in the SCW extraction at different time intervals

Time (min)		Total Carbohydrates (mg/g)					Total Phenolics (mg/g)				
		Sum of Squares	**df**	**Mean Square**	**F**	***p***	**Sum of Squares**	**df**	**Mean Square**	**F**	***p***
15	Between Groups	577.98	3.00	192.66	15.87	0.01	0.43	3.00	0.14	0.89	0.52
	Within Groups	48.57	4.00	12.14			0.64	4.00	0.16		
	Total	626.55	7.00				1.07	7.00			
30	Between Groups	1964.90	3.00	654.97	97.29	0.00	4.02	3.00	1.34	1.93	0.27
	Within Groups	26.93	4.00	6.73			2.77	4.00	0.69		
	Total	1991.82	7.00				6.78	7.00			
60	Between Groups	448.17	3.00	149.39	4.28	0.10	10.52	3.00	3.51	2.95	0.16
	Within Groups	139.50	4.00	34.88			4.76	4.00	1.19		
	Total	587.67	7.00				15.28	7.00			
m90	Between Groups	721.45	3.00	240.48	2.95	0.16	11.23	3.00	3.74	1.70	0.30
	Within Groups	325.83	4.00	81.46			8.78	4.00	2.20		
	Total	1047.28	7.00				20.01	7.00			
120	Between Groups	1013.07	3.00	337.69	3.09	0.15	9.15	3.00	3.05	1.64	0.31
	Within Groups	436.93	4.00	109.23			7.43	4.00	1.86		
	Total	1450.01	7.00				16.58	7.00			
150	Between Groups	1383.07	3.00	461.02	5.22	0.07	6.65	3.00	2.22	0.98	0.49
	Within Groups	353.09	4.00	88.27			9.09	4.00	2.27		
	Total	1736.16	7.00				15.74	7.00			
180	Between Groups	1309.09	3.00	436.36	3.64	0.12	5.95	3.00	1.98	1.02	0.47
	Within Groups	479.77	4.00	119.94			7.78	4.00	1.95		
	Total	1788.87	7.00				13.74	7.00			

(c) Static holding time variation: Effect of static holding time on the extraction.

Table C.5: Total carbohydrates and total phenolics at different static holding times in the SCW extraction (150°C, 15MPa and 2mL/min)

Static holding time (min)	Time (min)	Total Carbohydrates (mg/g)			Total Phenolics (mg/g)		
		1	2	Average	1	2	Average
10	15	25.70	30.36	28.03	3.25	3.12	3.19
	30	101.08	97.07	99.07	6.08	7.62	6.85
	60	135.83	142.07	138.95	11.51	14.35	12.93
	90	145.36	152.04	148.70	13.79	16.96	15.38
	120	153.62	159.64	156.63	15.05	18.14	16.59
	150	163.58	170.66	167.12	15.86	19.50	17.68
	180	174.32	181.83	178.08	16.45	19.92	18.18
20	15	16.07	17.72	16.89	1.22	1.37	1.30
	30	58.04	57.26	57.65	3.32	4.07	3.70
	60	113.59	121.71	117.65	11.55	11.98	11.77
	90	171.41	170.56	170.99	16.13	16.60	16.37
	120	218.92	219.21	219.06	17.75	19.33	18.54
	150	241.53	242.04	241.78	18.56	20.84	19.70
	180	255.54	255.32	255.43	19.07	21.87	20.47
30	15	12.03	15.86	13.95	1.36	1.19	1.28
	30	47.91	46.90	47.40	4.02	4.27	4.15
	60	125.39	127.87	126.63	11.78	12.92	12.35
	90	183.08	177.35	180.21	16.99	17.47	17.23
	120	216.86	214.15	215.51	19.85	19.98	19.91
	150	236.27	227.14	231.70	21.28	21.50	21.39
	180	247.64	237.70	242.67	22.30	22.42	22.36
60	15	8.52	15.33	11.93	1.25	1.60	1.42
	30	38.29	47.29	42.79	3.16	4.25	3.71
	60	137.13	137.10	137.12	10.07	11.59	10.83
	90	219.32	217.25	218.28	14.10	15.26	14.68
	120	261.21	258.50	259.85	17.51	18.08	17.80
	150	282.24	273.00	277.62	19.17	20.08	19.63
	180	291.75	281.13	286.44	19.64	20.91	20.27

Table C.6: ANOVA analysis to evaluate the effect of static holding time at different time intervals of the SCW extraction

Time (min)		Total Carbohydrates (mg/g)					Total Phenolics (mg/g)				
		Sum of Squares	**df**	**Mean Square**	**F**	***p***	**Sum of Squares**	**df**	**Mean Square**	**F**	***p***
15	Between Groups	309.45	3	103.150s	9.658	.026	5.179	3	1.726	72.380	.001
	Within Groups	42.72	4	10.680			.095	4	.024		
	Total	352.17	7				5.274	7			
30	Between Groups	3950.69	3	1316.898	106.753	.000	13.779	3	4.593	8.781	.031
	Within Groups	49.34	4	12.336			2.092	4	.523		
	Total	4000.04	7				15.871	7			
60	Between Groups	589.23	3	196.411	14.132	.014	4.815	3	1.605	1.083	.452
	Within Groups	55.59	4	13.898			5.930	4	1.483		
	Total	644.83	7				10.745	7			
90	Between Groups	5051.84	3	1683.947	163.429	.000	7.497	3	2.499	1.688	.306
	Within Groups	41.21	4	10.304			5.923	4	1.481		
	Total	5093.05	7				13.420	7			
m120	Between Groups	10831.05	3	3610.351	565.031	.000	11.593	3	3.864	2.496	.199
	Within Groups	25.56	4	6.390			6.193	4	1.548		
	Total	10856.61	7				17.786	7			
150	Between Groups	12725.39	3	4241.798	154.928	.000	13.802	3	4.601	1.905	.270
	Within Groups	109.517	4	27.379			9.662	4	2.416		
	Total	12834.91	7				23.464	7			
180	Between Groups	12469.15	3	4156.383	124.046	.000	17.489	3	5.830	2.168	.234
	Within Groups	134.027	4	33.507			10.754	4	2.689		
	Total	12603.18	7				28.243	7			

(d) Particle size variation: Effect of different particle size on the SCW extraction.

Table C.7: Total carbohydrates and total phenolics at different milling extents (150°C, 15MPa, 2mL/min and 60 min of static holding time)

Milling extent	Time (min)	Total Carbohydrates (mg/g)			Total Phenolics (mg/g)		
		1	2	Average	1	2	Average
1 time	15	16.38	16.17	16.27	1.38	1.20	1.29
	30	55.23	58.44	56.83	4.08	3.40	3.74
	60	109.28	113.02	111.15	12.01	12.37	12.19
	90	166.26	171.37	168.81	16.67	14.77	15.72
	120	215.35	218.64	217.00	19.40	16.59	17.99
	150	236.72	240.69	238.70	20.92	17.37	19.14
	180	250.13	251.69	250.91	21.94	18.62	20.28
2 times	15	16.95	20.00	18.48	1.00	0.85	0.93
	30	60.33	56.77	58.55	2.47	2.23	2.35
	60	113.78	107.90	110.84	4.44	4.54	4.49
	90	172.00	168.61	170.30	12.54	13.23	12.89
	120	218.41	202.67	210.54	17.48	17.71	17.60
	150	241.48	226.88	234.18	20.73	20.57	20.65
	180	255.09	237.34	246.22	22.33	22.06	22.19
3 times	15	19.26	14.64	16.95	1.49	1.18	1.33
	30	61.01	53.06	57.03	4.69	4.31	4.50
	60	124.86	119.89	122.38	11.56	9.77	10.66
	90	173.75	173.66	173.71	17.50	14.14	15.82
	120	219.22	215.13	217.18	20.07	17.50	18.79
	150	241.35	241.34	241.35	21.47	19.60	20.54
	180	255.37	255.97	255.67	22.52	20.74	21.63

Table C.8: ANOVA analysis to evaluate the effect of extent of milling at different time intervals of the SCW extraction

Time (min)		Total Carbohydrates (mg/g)					Total Phenolics (mg/g)				
		Sum of Squares	**df**	**Mean Square**	**F**	***p***	**Sum of Squares**	**df**	**Mean Square**	**F**	***p***
15	Between Groups	5.101	2	2.550	.499	.650	.201	2	.101	3.956	.144
	Within Groups	15.318	3	5.106			.076	3	.025		
	Total	20.418	5				.278	5			
30	Between Groups	3.513	2	1.756	.122	.889	4.749	2	2.375	21.753	.016
	Within Groups	43.124	3	14.375			.327	3	.109		
	Total	46.637	5				5.077	5			
60	Between Groups	172.847	2	86.423	7.071	.073	66.550	2	33.275	59.455	.004
	Within Groups	36.666	3	12.222			1.679	3	.560		
	Total	209.513	5				68.229	5			
90	Between Groups	25.137	2	12.569	2.002	.280	11.103	2	5.552	2.173	.261
	Within Groups	18.838	3	6.279			7.664	3	2.555		
	Total	43.975	5				18.767	5			
120	Between Groups	57.153	2	28.576	.623	.594	1.469	2	.735	.303	.759
	Within Groups	137.670	3	45.890			7.270	3	2.423		
	Total	194.823	5				8.739	5			
150	Between Groups	52.547	2	26.273	.689	.567	2.811	2	1.405	.521	.640
	Within Groups	114.384	3	38.128			8.097	3	2.699		
	Total	166.931	5				10.908	5			
180	Between Groups	89.378	2	44.689	.844	.512	3.871	2	1.936	.812	.523
	Within Groups	158.893	3	52.964			7.153	3	2.384		
	Total	248.271	5				11.024	5			

APPENDIX D

SUBCRITICAL AQUEOUS ETHANOL EXTRACTION (Refer to Table 4.5)

(a) Total carbohydrates and total phenolics: The quadratic model was significant for total carbohydrates and total phenolics analysis.

Table D.1: Experimental and predicted values for total carbohydrates by RSM

Run	Total Carbohydrates (mg/g)		
	Experimental Value	Predicted value	Residual value
1	194.7	198.1	-3.4
2	373.4	388.5	-15.1
3	180.1	199.4	-19.3
4	376.8	365.4	11.4
5	207.8	164.0	43.8
6	334.3	349.6	-15.3
7	194.8	251.4	-56.6
8	397.4	412.6	-15.2
9	195.0	197.9	-2.9
10	451.7	416.9	34.8
11	213.1	219.6	-6.5
12	352.3	414.2	-61.9
13	86.2	119.4	-33.2
14	334.8	333.6	1.2
15	224.2	227.2	-3.0
16	398.6	417.1	-18.4
17	110.6	90.0	20.6
18	489.5	470.2	19.3
19	209.8	234.7	-24.9
20	384.2	319.4	64.8
21	413.0	401.4	11.6
22	398.4	370.1	28.3
23	238.6	223.7	14.9
24	252.9	227.9	25.0
25	428.7	424.7	4.0
26	428.1	424.7	3.4
27	410.1	424.7	-14.6
28	422.4	424.7	-2.3
29	428.9	424.7	4.2
30	430.0	424.7	5.3

Table D.2: Experimental and predicted values for total phenolics by RSM

Run	Total Phenolics (mg/g)		
	Actual value	**Predicted value**	**Residual value**
1	12.9	14.7	-1.8
2	31.9	41.1	-9.2
3	13.3	17.5	-4.2
4	32.1	37.2	-5.1
5	11.7	13.4	-1.7
6	28.4	36.2	-7.8
7	14.5	22.0	-7.5
8	27.4	38.1	-10.7
9	22.6	18.6	4.0
10	46.0	49.0	-3.0
11	24.3	27.1	-2.7
12	45.7	50.8	-5.1
13	13.6	19.1	-5.4
14	43.3	45.8	-2.5
15	35.7	33.3	2.4
16	44.7	53.4	-8.7
17	10.8	11.0	-0.1
18	74.8	57.5	17.4
19	28.0	22.9	5.1
20	45.4	33.3	12.1
21	39.6	34.6	4.9
22	48.3	36.0	12.3
23	37.5	22.1	15.4
24	43.2	41.4	1.9
25	45.3	45.3	0.0
26	43.3	45.3	-2.0
27	46.0	45.3	0.7
28	45.4	45.3	0.1
29	45.6	45.3	0.3
30	46.3	45.3	1.0

APPENDIX E

SUBCRITICAL AQUEOUS ETHANOL EXTRACTION:

(15 MPa, 12% ethanol concentration, 5mL/min, 15 min static time, and total extraction time of 20 min)

Table E.1: Total carbohydrates and total phenolics of the sCF extract solutions (n=2)

Temperature (°C)	Total Carbohydrates (mg/g)				Total Phenolics (mg/g)			
	Run 1	Run 2	Average	SD	Run 1	Run 2	Average	SD
100	88.34	96.07	92.21	5.46	4.58	7.56	6.07	2.10
160	323.13	408.64	365.89	60.46	40.59	41.95	41.27	0.96
180	421.80	449.8	435.80	19.80	79.85	71.02	75.43	6.24
200	486.32	516.1	501.21	21.06	113.08	102.75	107.92	7.31
220	554.48	541.08	547.78	9.48	123.03	136.02	129.53	9.18
240	585.29	618.25	601.77	23.30	114.76	125.54	120.15	7.62
260	365.32	349.56	357.44	11.14	97.87	97.48	97.67	0.27

Table E.2: pH, RI and conductivity of the sCF extract solutions (single run)

Temperature (°C)	pH	RI	Conductivity (µs/cm)
100	7.96	1.3337	145.66
160	5.45	1.3401	335.45
180	4.96	1.3423	985.68
200	4.01	1.3467	1248.50
220	3.56	1.3569	1345.80
240	3.05	1.3557	989.46
260	5.48	1.3405	754.44

Table E.3: Scavenging activity using DPPH analysis (single run)

Temperature (°C)	Scavenging Activity		
	Absorbance 1	Absorbance 2	% DPPH
100	0.598	0.598	32.4
160	0.260	0.268	70.0
180*	0.383	0.355	72.3
200	0.112	0.125	86.5
220	0.103	0.105	88.3
240	0.099	0.899	89.3
260	0.216	0.216	75.4

*This experiment was conducted and analysed in other month.

Table E.4: Ferrous sulphate equivalent for the extract solutions (single run)

Temperature (°C)	FRAP (mMol Ferrous sulphate reduction/g of sample)			
	Analysis 1	Analysis 2	Average	SD
100	29.67	28.89	29.28	0.55
160	360.08	354.42	357.25	4.00
180	440.36	416.92	428.64	16.58
200	519.22	520.45	519.84	0.87
220	544.48	558.25	551.37	9.74
240	569.44	569.57	569.51	0.09
260	181.49	179.47	180.48	1.43

APPENDIX F

SUBCRITICAL FLUID AQUEOUS ETHANOL EXTRACTION: variation in pH of the solvent

(240°C, 12% ethanol concentration, 5mL/min and 15 min static time)

Table F.1: Total carbohydrates content at high pH (11), neutral pH (7) and low pH (3) of sCF aqueous ethanol solvent

Time	Total Carbohydrates (mg/g)											
	pH 3				pH 7				pH 11			
	Run 1	Run 2	Average	SD	Run 1	Run 2	Average	SD	Run 1	Run 2	Average	SD
4 min	112.1	113.0	112.6	0.7	108.3	100.9	104.6	5.19	122.3	117.0	119.7	3.7
8 min	248.8	250.6	249.7	1.3	253.6	250.9	252.2	1.90	260.4	252.7	256.6	5.5
12 min	320.4	308.0	314.2	8.7	412.7	412.7	412.7	0.01	393.1	385.8	389.4	5.1
16 min	363.4	342.7	353.1	14.6	559.5	545.2	552.3	10.10	465.3	455.6	460.4	6.9
20 min	396.9	372.8	384.8	17.0	592.3	586.8	589.6	3.90	542.2	529.4	535.8	9.1
24 min	408.3	387.4	397.9	14.8	609.8	604.9	607.3	3.48	578.6	570.0	574.3	6.1
28 min	410.9	391.2	401.0	13.9	622.9	617.0	620.0	4.17	606.1	595.6	600.9	7.4
32 min	412.3	394.0	403.1	12.9	635.3	619.3	627.3	11.30	608.4	597.9	603.1	7.4

Table F.2: Total phenolics content at high pH (11), neutral pH (7) and low pH (3) of aqueous ethanol solvent

Time	Total Phenolics (mg/g)											
	pH 3				pH 7				pH 11			
	Run 1	Run 2	Average	SD	Run 1	Run 2	Average	SD	Run 1	Run 2	Average	SD
4 min	16.9	17.0	17.0	0.05	13.5	12.5	13.0	39.11	17.3	16.7	17.0	0.4
8 min	42.0	41.9	41.9	0.08	79.9	81.6	80.7	11.31	43.1	42.7	42.9	0.3
12 min	65.9	62.5	64.2	2.39	98.7	101.6	100.2	7.10	67.8	66.3	67.1	1.1
16 min	83.5	80.7	82.1	1.96	111.3	113.2	112.2	5.95	91.6	89.0	90.3	1.9
20 min	101.0	97.5	99.3	2.44	121.5	123.3	122.4	4.37	113.2	111.6	112.4	1.2
24 min	112.6	107.3	110.0	3.70	128.8	130.6	129.7	1.73	130.2	126.8	128.5	2.4
28 min	121.5	117.1	119.3	3.08	131.1	133.0	132.0	1.48	146.5	142.7	144.6	2.7
32 min	126.2	124.2	125.2	1.42	132.9	134.7	133.8	1.40	160.3	152.0	156.1	5.9

Table F.3: pH, RI and conductivity of the extracts solutions

	pH 3			pH 7			pH 11		
Time	**pH**	**RI**	**Conductivity (µs/cm)**	**pH**	**RI**	**Conductivity (µs/cm)**	**pH**	**RI**	**Conductivity (µs/cm)**
4 min	4.13	1.34084	629.2	3.41	1.34486	642.5	3.18	1.34126	481.3
8 min	4.23	1.34098	1345	3.45	1.33866	889.4	3.64	1.34946	1586
12 min	4.68	1.34012	988.6	4.88	1.34855	1088.1	4.64	1.34012	828.8
16 min	4.73	1.33956	758.4	4.65	1.34158	985.5	4.98	1.33956	564.2
20 min	5.01	1.33714	451.4	4.99	1.34115	667.7	5.77	1.33714	494.2
24 min	5.04	1.33396	374.2	5.08	1.33955	442.2	6.11	1.33396	396.4
28 min	5.08	1.33345	317.4	5.88	1.33956	348.9	6.30	1.33345	214.9
32 min	5.71	1.33344	254.5	6.44	1.33855	167.4	6.11	1.33344	201.1

APPENDIX G

KINETIC DATA AT THE OPTIMAL CONDITIONS FOR SUBCRITICAL AQUEOUS ETHANOL EXTRACTION

Table G.1: Kinetics of total carbohydrates and total phenolics (240°C, 12% ethanol concentration, 5mL/min and 15 min static time)

Time (min)	Total Carbohydrates (mg/g)				Total Phenolics (mg/g)			
	1	2	Average	S.D	1	2	Average	S.D.
4	108.3	100.9	104.6	5.19	13.5	12.5	13.0	39.11
8	253.6	250.9	252.2	1.90	79.9	81.6	80.7	11.31
12	412.7	412.7	412.7	0.01	98.7	101.6	100.2	7.10
16	559.5	545.2	552.3	10.10	111.3	113.2	112.2	5.95
20	592.3	586.8	589.6	3.90	121.5	123.3	122.4	4.37
24	609.8	604.9	607.3	3.48	128.8	130.6	129.7	1.73
28	622.9	617.0	620.0	4.17	131.1	133.0	132.0	1.48
32	635.3	619.3	627.3	11.30	132.9	134.7	133.8	1.40
36	638.1	622.2	630.2	11.27	134.3	136.3	135.3	1.39
40	640.2	623.6	631.9	11.73	135.5	137.6	136.5	1.31
44	642.1	625.6	633.9	11.68	136.5	138.5	137.5	1.25
48	643.8	627.5	635.7	11.53	137.5	139.4	138.4	1.19
52	645.7	628.9	637.3	11.87	138.2	140.1	139.1	1.11
56	647.1	630.2	638.6	11.91	138.7	140.5	139.6	1.06
60	648.4	631.3	639.9	12.10	139.2	141.0	140.1	1.05
64	649.3	632.1	640.7	12.15	139.7	141.5	140.6	1.03
68	650.2	632.9	641.5	12.20	140.1	141.8	140.9	1.03
72	650.7	633.5	642.1	12.17	140.4	142.2	141.3	1.02
76	651.3	634.1	642.7	12.16	140.8	142.5	141.7	0.99
80	651.7	634.5	643.1	12.14	141.3	142.9	142.1	0.95
84	652.2	635.0	643.6	12.16	141.7	143.3	142.5	0.93
88	652.4	635.3	643.9	12.09	142.1	143.6	142.9	0.90
92	652.7	635.7	644.2	12.06	142.5	144.0	143.3	0.84
96	653.0	636.0	644.5	12.06	143.0	144.4	143.7	0.77
100	653.3	636.2	644.8	12.07	143.4	144.7	144.1	0.77
104	653.6	636.5	645.1	12.07	143.8	145.1	144.5	0.78
108	653.9	636.8	645.3	12.08	144.2	145.5	144.8	0.78
112	654.2	637.1	645.6	12.07	144.5	145.8	145.2	0.78
116	654.4	637.4	645.9	12.07	144.8	146.1	145.5	0.76
120	654.7	637.6	646.2	12.07	145.0	146.3	145.7	0.91

Table G.2: Kinetics of total carbohydrates and total phenolics (220°C, 12% ethanol concentration, 5mL/min and 15 min static time)

Time (min)	Total Carbohydrates (mg/g)				Total Phenolics (mg/g)			
	1	2	Average	S.D	1	2	Average	S.D.
4	94.0	81.6	87.8	8.7	14.7	13.2	14.0	1.1
8	240.3	241.9	241.1	1.2	73.7	64.6	69.1	6.4
12	388.9	402.4	395.7	9.5	92.0	84.5	88.2	5.3
16	536.0	528.1	532.0	5.6	107.3	100.2	103.8	5.0
20	562.7	556.9	559.8	4.1	114.0	107.9	111.0	4.3
24	578.7	571.4	575.0	5.1	121.8	115.8	118.8	4.2
28	585.4	585.2	585.3	0.2	125.2	119.3	122.2	4.2
32	587.7	588.1	587.9	0.3	126.8	121.0	123.9	4.1
36	590.2	590.4	590.3	0.1	128.0	122.2	125.1	4.1
40	592.1	591.8	592.0	0.2	129.0	123.2	126.1	4.1
44	593.9	593.8	593.8	0.1	129.8	123.9	126.9	4.2
48	595.4	595.3	595.3	0.1	130.6	125.0	127.8	4.0
52	596.8	596.6	596.7	0.2	131.0	125.3	128.2	4.0
56	598.1	597.8	597.9	0.2	131.4	125.7	128.6	4.0
60	599.1	598.9	599.0	0.1	131.7	126.1	128.9	4.0
64	600.1	600.0	600.1	0.1	132.1	126.5	129.3	4.0
68	601.1	601.1	601.1	0.0	132.4	126.8	129.6	4.0
72	601.9	602.1	602.0	0.1	132.8	127.1	130.0	4.0
76	602.6	603.1	602.8	0.3	133.1	127.4	130.3	4.0
80	602.9	603.9	603.4	0.7	133.4	127.8	130.6	4.0
84	603.2	604.6	603.9	1.0	133.8	128.1	131.0	4.0
88	603.5	605.1	604.3	1.1	134.1	128.5	131.3	4.0
92	603.8	605.2	604.5	1.0	134.4	128.7	131.5	4.0
96	604.1	605.4	604.7	0.9	134.6	128.9	131.8	4.0
100	604.4	605.5	604.9	0.8	134.8	129.2	132.0	4.0
104	604.7	605.7	605.2	0.7	135.0	129.3	132.1	4.0
108	604.9	605.8	605.4	0.6	135.1	129.5	132.3	4.0
112	605.2	606.0	605.6	0.5	135.3	129.7	132.5	4.0
116	605.5	606.2	605.8	0.4	135.5	129.9	132.7	4.0
120	605.8	606.3	606.1	0.4	135.6	130.0	132.8	4.0